Neutral Amino Acid Transport at the Human Blood-Brain Barrier*

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Katherine M. Hargreaves‡ and William M. Pardridge§
From the Department of Medicine and Brain Research Institute, UCLA School of Medicine, Los Angeles, California 90024

The kinetics of human blood-brain barrier neutral amino acid transport sites are described using isolated human brain capillaries as an in vitro model of the human blood-brain barrier. Kinetic parameters of transport (Km, Vmax, and Kp) were determined for eight large neutral amino acids. Km values ranged from 0.30 ± 0.08 μM for phenylalanine to 8.8 ± 4.6 μM for valine. The amino acid analogs N-methylaminoisobutyric acid and 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid were used as model substrates of the alanine- and leucine-preferring transport systems, respectively. Phenylalanine is transported solely by the L-system (which is sensitive to p-chloromercuribenzenesulfonic acid) and leucine is transported equally by the L- and ASC-system (which is sodium-dependent and N-methylaminoisobutyric acid-independent). Dose-dependent inhibition of the high affinity transport system by p-chloromercuribenzenesulfonic acid is demonstrated for phenylalanine, similar to the known sensitivity of blood-brain barrier transport in vivo. The Km values for the human brain capillary in vitro correlate significantly (r = 0.83, p < 0.01) with the Km values for the rat brain capillary in vivo. The results show that the affinity of human blood-brain barrier neutral amino acid transport sites is very low compared to plasma amino acid concentrations. This provides a physical basis for the selective vulnerability of the human brain to derangements in amino acid availability caused by a selective hyperaminoacidaemia, e.g. hyperphenylalaninemia.

The brain is known to be acutely sensitive to changes in plasma amino acid concentrations within the physiologic range (1, 2). Examples of this sensitivity are the inhibition in cerebral protein synthesis caused by hyperphenylalaninemia and the reversal of this inhibition by the co-administration of other large neutral amino acids that compete with phenylalanine for transport into the brain from blood (3). Similarly, the carbohydrate-induced increase in brain tryptophan and serotonin concentrations is caused by insulin-mediated hypoaminoacidemia and decreased competition for transport into brain between circulating tryptophan and other neutral amino acids (1). Elevated plasma concentrations of branched-chain amino acids in portacaval shunted rats (4) or in diabetic rats (5) and altered plasma amino acid concentrations in response to dietary protein (6, 7) have also been shown to alter brain amino acid availability.

The rate-limiting step of amino acid transport into the brain is at the blood capillary wall, i.e. the blood-brain barrier (BBB) (8). It is known, at least in laboratory rats, that the physical basis of the unique sensitivity of the brain to changes in plasma amino acid concentrations within the physiologic range arises from the uniquely high affinity (low Km) of BBB neutral amino acid transport (8). For example, the Km of neutral amino acid transport through the BBB in rats is of the order of 10–100 μM, which approximates the normal physiologic concentration of amino acids (9). Conversely, the Km of neutral amino acid transport into other organs of the rat is of the order of 1–10 mM or even greater (10).

The extrapolation of BBB amino acid transport paradigms developed in rats to humans requires that the transport of amino acids across the human BBB is also characterized by a system with a very high affinity, i.e. low Km values. Recently, it has been shown that the capillary isolated from fresh autopsied human brain can be used as an in vitro model system of the human BBB and that Km values for phenylalanine transport obtained with the isolated human or rat brain capillaries are approximately the same (11). Estimates for the Km of transport of the other large neutral amino acids that compete with phenylalanine for transport through the human BBB must still be determined. In addition to allowing for further characterization of the transport system in humans, the estimation of the Km values for the other neutral amino acids allows for quantitative predictions of changes in amino acid availability in human brain from an analysis of amino acid ratios in human blood. Since the Km of neutral amino acid transport in the rat varies over a 10-fold range (9, 10), it is important to normalize plasma amino acid ratios by weighting each amino acid concentration by the respective Km value (12). Therefore, these studies were designed to assess the kinetics of transport of phenylalanine and seven other neutral amino acids (tyrosine, isoleucine, leucine, valine, tryptophan, histidine, and methionine) using isolated human brain capillaries at an in vitro model system of the human BBB.

EXPERIMENTAL PROCEDURES

Materials—The following tritiated amino acids were obtained from Du Pont-New England Nuclear: L-[3,4,5-3H]leucine (140.5 Ci/mmol), L-[ring-2,5-3H]histidine (51 Ci/mmol), L-[3,4-3H]valine (64.6 Ci/mmol), L-[4,5-3H]isoleucine (127.0 Ci/mmol), L-[5-3H]tryptophan (20 Ci/mmol), and L-[methyl-3H]methionine (50 Ci/mmol); or from Amersham Corp.: L-[2,3,5,6-3H]tyrosine (114 Ci/mmol) and L-[2,3,5,6-3H]phenylalanine (88 Ci/mmol). [U-14C]Sucrose (671 mCi/mmol) and

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§ To whom reprint requests should be addressed.

1 The abbreviations used are: BBB, blood-brain barrier; NMAIB, N-methylaminoisobutyric acid; BCH, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; pCMBS, p-chloromercuribenzenesulfonic acid; mg, milligram protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L-system, leucine-preferring system; ASC-system, alanine-serine-cysteine-preferring system.
[H]water (25 mCi/g) were also obtained from Du Pont-New England Nucleon. Unlabeled amino acids, bovine serum albumin, N-aminobutyric acid (NMAIB), and p-chloromercuribenzenesulfonic acid (pCMBS) were obtained from Sigma.

Autopsy Data—Brains were obtained from six autopsies over a 1-year period. Patients included five males ranging from 15 to 79 years of age and one female of 93 years. Causes of death were cardiomyopathy, pancreatic cancer, bladder cancer, or respiratory arrest. Brains were obtained between 10 and 30 h postmortem.

Microvessel Isolation—Capillaries were isolated by mechanical homogenization of cortex obtained from fresh human brain as described previously (13, 14) and stored in liquid nitrogen at -70 °C in 0.28 M sucrose, 0.02 mM Tris, 2 mM dithiothreitol. Initial studies showed no appreciable change in the saturation plot obtained with cryopreserved microvessels as compared to capillaries freshly isolated from the same brain (Fig. 1).

Amino Acid Transport—Capillaries were thawed at 37 °C, pelleted at 1500 x g for 10 min, and resuspended in 10 mM HEPES-buffered Ringer's solution (141 mM NaCl, 4 mM KCl, 2.8 mM CaCl₂, 10 mM HEPES, 1 mM MgSO₄, 10 mM D-glucose, pH 7.4) containing 0.1% bovine serum albumin. Incubations were performed at room temperature for 2 min in the presence of 2 µCi of [3H]amino acid, 0.4 µCi of [14C]sucrose, and 0–500 µM unlabeled amino acid (11). BCH (10 mM), NMAIB (10 mM), and pCMBS (0.1–1.0 mM) were preincubated with capillaries for 15 min at 23 °C prior to addition of isotope. The incubation mixtures contained approximately 100 µg of capillary protein. The incubation was stopped by the addition of 4 ml of ice-cold HEPES-buffered Ringer's solution and immediately poured over Whatman GF/C filters in a Millipore filtration apparatus attached to a vacuum pump. The filters were washed with an additional 10 ml of ice-cold buffer, placed in 1 ml of 1 N NaOH, and incubated at 60 °C for 1 h. An aliquot of dissolved capillaries was removed for protein determination (15). The remaining material was suspended in 10 ml of Scint-A (Packard Instrument Co.) and counted in a Packard 3390 Tri-Carb liquid scintillation spectrometer. All counts were corrected to disintegrations/minute by standard quench correction curves. The amino acid volume of distribution (V₀) in microliters/mg, was calculated as follows.

\[ V₀ = \frac{[3H] \text{dpm}}{\mu\text{g} \text{medium}} - \frac{[14C] \text{dpm}}{\mu\text{g} \text{medium}} \]

The clearance (Cl) in microliters/minute/mg, was computed from Cl = V₀/t, where t = 2 min.

Transstimulation experiments were performed by preincubating capillaries in the presence or absence (control) of 50 µM amino acid for 60 min at 23 °C. The transport assay was performed by the addition of 50 µl of this capillary suspension to 950 µl of fresh buffer containing isotope. Since the initial buffer containing 50 µM amino acid underwent a 20-fold dilution, each amino acid was present at 2.5 µM during the final 2 min period employed to measure [H]phenylalanine and [14C]sucrose uptake.

Parameter Estimation—The clearance values were fit to the following equation:

\[ Cl = \frac{V_{\text{max}}}{K_m + S} + K_D \]

where \( V_{\text{max}} \) is the maximal transport rate, \( K_m \) is the half-saturation constant, \( K_D \) is the nonsaturable constant, and S is the medium amino acid concentration. The parameters were estimated by nonlinear regression analysis using subroutine P3R of the BMDP statistical software package on an IBM PC-XT microcomputer (16). Standard errors were proportional to means; therefore, the data were weighted by 1/Cl² (11). Since the nonsaturable route of transport was believed to be a second low affinity transport system (L), some data sets were fit to a function where \( K_{mL} = V_{\text{maxL}}/(K_{mS} + S) \), so that \( V_{\text{max}} \) and \( K_m \) values were estimated for both the high and low affinity transport systems (11).

RESULTS

The transport of [H]tryptophan into isolated human brain capillaries is shown in Fig. 2 over concentration ranges from 0.2 to 10 µM and from 10 to 500 µM. These data were analyzed by the three-parameter \( (V_{\text{max}}, K_m, K_D) \) model described

![Fig. 1. Clearance of phenylalanine in fresh and cryopreserved human capillaries obtained from the same brain. Incubations were performed at room temperature for 2 min in the presence of 0.4 µCi of [14C]sucrose, 2.0 µCi of [H]phenylalanine, and ~100 µg of capillary protein. Each point represents the mean ± S.E. of three replicates.](image)

![Fig. 2. [H]Tryptophan clearance in human brain capillaries. Incubations were performed at room temperature for 2 min in the presence of 0.4 µCi of [14C]sucrose, 2.0 µCi of [H]tryptophan, and ~100 µg of human capillary protein. Each point represents the mean ± S.E. of three replicates.](image)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>( K_m ) (µM)</th>
<th>( V_{\text{max}} ) (pmol/min·mg)</th>
<th>( K_D ) (µl/min·mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe</td>
<td>0.30 ± 0.08</td>
<td>6.2 ± 0.8</td>
<td>0.66 ± 0.04</td>
</tr>
<tr>
<td>Ile</td>
<td>1.3 ± 0.4</td>
<td>11.9 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Trp</td>
<td>2.7 ± 1.1</td>
<td>7.6 ± 1.9</td>
<td>NS</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.0 ± 0.7</td>
<td>15.6 ± 2.9</td>
<td>0.57 ± 0.05</td>
</tr>
<tr>
<td>Leu</td>
<td>3.3 ± 0.7</td>
<td>15.4 ± 2.3</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td>Met</td>
<td>5.1 ± 2.5</td>
<td>14.8 ± 6.2</td>
<td>0.50 ± 0.10</td>
</tr>
<tr>
<td>His</td>
<td>5.1 ± 2.0</td>
<td>38.0 ± 12.8</td>
<td>0.64 ± 0.16</td>
</tr>
<tr>
<td>Val</td>
<td>8.8 ± 4.6</td>
<td>13.3 ± 6.4</td>
<td>0.72 ± 0.08</td>
</tr>
</tbody>
</table>

*NS, not significantly different from zero.
FIG. 3. Competition for \(^{3}H\)leucine uptake in human brain capillaries. Amino acids were present individually at concentrations of 50 \(\mu M\). Incubations were performed at room temperature for 2 min and were initiated by the addition of microvessels. Values represent means \(\pm S.E.\) of three replicates.

under “Experimental Procedures,” yielding \(K_m = 2.9 \pm 0.7 \mu M\) and \(V_{max} = 15.6 \pm 2.9 \text{ pmol/min \cdot mg}\), for the high affinity system and \(K_d = 0.57 \pm 0.05 \text{ \mu l/min \cdot mg}\), for the low affinity system (Table I). These data were also fit to a four-parameter model for both high and low affinity transport systems (see “Experimental Procedures”), and the data fits yielded the following parameters: \(K_d^H = 2.0 \pm 0.6 \mu M\), \(V_{max}^H = 11.2 \pm 2.8 \text{ pmol/min \cdot mg}\), \(K_d^L = 803 \pm 550 \mu M\), and \(V_{max}^L = 582 \pm 210 \text{ pmol/min \cdot mg}\). \(V_{max}^H/K_d^L = 0.72\), which approximates the estimated \(K_d\) shown in Table I. Saturation studies were also performed for seven other neutral amino acids, and the clearance values are given in Table II. These clearance data were fit to the three-parameter model (see “Experimental Procedures”) to provide the \(K_m\), \(V_{max}\), and \(K_d\) values shown in Table I.

The effects of the physiologic concentration (50 \(\mu M\)) of other neutral amino acids on the transport of \(^{3}H\)leucine into human brain capillaries are shown in Fig. 3. An acidic amino acid (e.g. glutamic acid), a basic amino acid (e.g. arginine), or an imino acid (e.g. proline) had no significant inhibition on 

FIG. 4. Characterization of leucine and phenylalanine transport. Values represent means \(\pm S.E.\) of three replicates. Human capillaries (~100 \(\mu g\) of protein) were preincubated at room temperature for 15 min in the presence of inhibitor. Sodium chloride (\(Na^+\)) or choline chloride (\(Ch\)) was present in the 10 mM HEPES-buffered Ringer’s solution incubation medium. NMAIB and BCH were present at 10 mM. \(NS\), nonsaturable component of transport. Upper, clearance of leucine and phenylalanine in the absence of NMAIB, the absence of sodium, and the presence of BCH; lower, clearance data (upper) expressed as percent of total transport by A (sodium-dependent, NMAIB-sensitive), L (sodium-independent, BCH-sensitive), and \(NS\) (nonsaturable)-system transport processes.
alanine also had a marked inhibitory effect on \(^{3}H\)leucine transport.

The inhibition of \(^{3}H\)leucine transport into human brain capillaries by small neutral amino acids, such as glycine or alanine, was unexpected since previous studies have shown that glycine and alanine at 50 \(\mu M\) do not inhibit \(^{3}H\)phenylalanine transport into human brain capillaries (11). Therefore, the possibility of multiple transport systems for neutral amino acids such as leucine versus phenylalanine was investigated. In Fig. 4 (upper), the effects of 10 \(\mu M\) NMAIB (a specific inhibitor of the alanine-preferring neutral amino acid transport system) and 10 \(\mu M\) BCH (a specific inhibitor of the leucine-preferring amino acid transport system) (17) were investigated for both \(^{3}H\)leucine and \(^{3}H\)phenylalanine transport into human brain capillaries. In addition, the effects of replacing sodium with stoichiometric amounts of choline were also evaluated since the alanine-preferring system is sodium-dependent and the leucine-preferring system is sodium-independent (17). The data in Fig. 4 (upper) are replotted in Fig. 4 (lower) as a percent of control, and the data show that phenylalanine is taken up essentially by a system that is inhibited only by BCH, whereas leucine is transported nearly equally by the BCH-sensitive system (i.e. the L-system) and by the sodium-dependent system that is not inhibited by NMAIB (i.e. the alanine-serine-cysteine (ASC)-preferring system) (17).

Since BBB neutral amino acid transport in vivo is known to be acutely sensitive to the sulfhydryl reagent pCMBS (18), the effects of this reagent on \(^{3}H\)phenylalanine transport into isolated human brain capillaries were investigated (Fig. 6). These data show that phenylalanine transport is inhibited by pCMBS, with a \(K_{i}\) of approximately 0.4 \(\mu M\).

The relationship between the \(K_{m}\) values reported in Table I for the human BBB and the previously reported \(K_{m}\) values for the rat BBB (9) determined in vivo is shown in Fig. 7. These data show a significant correlation between the \(K_{m}\) values of the two species; however, the \(K_{m}\) for the human BBB is about 8–40-fold lower than the corresponding \(K_{m}\) values for the rat BBB in vivo.

**DISCUSSION**

These experiments suggest the following conclusions. First, the kinetics of neutral amino acid transport at the human BBB may be estimated using the isolated brain capillary, and the \(K_{m}\) and \(V_{max}\) values in Table I represent the first estimates of these values for the human BBB. Second, aromatic amino acids such as phenylalanine are transported essentially by a single BCH-sensitive system, whereas branched-chain amino acids such as leucine are transported equally by a BCH-
sensitive system and by a sodium-dependent, NMAIB-independent system (Fig. 4).

The above conclusions are based on the assumption that brain capillaries isolated from post-mortem human brain may be used as an in vitro model system of the human BBB. Several lines of evidence support the validity of this assumption. First, previous studies have shown that the human brain capillary retains several functions found in vivo, such as Factor VIII antigen expression, γ-glutamyl transpeptidase activity, and avid binding of insulin (14), insulin-like growth factors (19), or transferrin (20) to specific saturable receptors. In addition, the kinetics of phenylalanine transport into human brain capillaries are comparable to those for capillaries isolated from fresh rat brain (11). However, caution must be used in using the isolated human brain capillary as a model system of the BBB in vivo since the isolated human brain capillary is not metabolically normal. For example, the human brain capillary is leaky, e.g., the cell takes up trypan blue (13, 14), and the [³H]water space is not significantly different from the [¹⁴C]sucrose space.  Other studies (21, 22) have shown that brain capillaries isolated by either a mechanical or enzymatic homogenization CMBS, after correcting for the sucrose volume, which are used as an in vitro model system of the human BBB. The extrapolation of these generalizations must be used with caution.

Finally, the Kₗ values in Table I allow for quantitative estimates of amino acid availability in human brain based on an analysis of plasma amino acid ratios. For example, owing to competition effects at BBB transport sites, the concentration of tryptophan in brain is determined by the tryptophan neutral amino acid ratio. The regular tryptophan ratio is defined (1) as: Trp ratio = (Trp/Σ LNAA)ₗ, where Trp is the plasma tryptophan concentration, and Σ LNAA is the sum of concentrations of competing large neutral amino acids. This calculation assumes that all large neutral amino acids have the same affinity for the transport system. However, the data in Table I indicate that phenylalanine has nearly a 30-fold greater affinity for this system than does valine. When large neutral amino acids concentrations change proportionally, e.g., after a meal, then the use of the regular tryptophan ratio is probably valid. However, when there is selective hyperaminoacidemia, e.g. hyperphenylalaninemia, then computation of regular amino acid ratios significantly underestimates the change in amino acid availability caused by the hyperphenylalaninemia (12). The Kₗ normalized ratio more accurately predicts changes in amino acid availability (12), where

\[
Kₗ \text{ normalized } \text{Trp ratio} = \frac{\text{Trp}}{\sum \text{LNAA}} \quad K^{\text{Trp}}
\]

where K²ⁱ represents the Kₗ of tryptophan, and K²ⁱ LNAA is the Kₗ of the competing large neutral amino acid. Kₗ normalized ratios may now be computed for humans using the Kₗ values described in Table I.

In summary, these studies provide estimates of the Kₗ of neutral amino acid transport into isolated human brain capillaries, which are used as an in vitro model of the human BBB. The extrapolation of these Kₗ values to the in vivo condition must be done with caution since the values are considerably lower than those found in vivo (Fig. 7). Nevertheless, the rank order observed in the human brain capillary correlates with the rate BBB in vivo (Fig. 7). Therefore, these Kₗ values should reliably predict competition effects occurring at the human BBB in association with alterations in plasma concentrations of large neutral amino acids.

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REFERENCES

5 K. M. Hargreaves and W. M. Pardridge, unpublished observations.
Brain Capillary Amino Acid Transport