Na⁺-dependent transport of large neutral amino acids occurs at the abluminal membrane of the blood-brain barrier

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CEREBRAL CAPILLARIES have more extensive tight junctions between endothelial cells than other capillaries (7). This arrangement creates a blood-brain barrier (BBB) by blocking paracellular movement. It also prevents the movement of intrinsic proteins and lipids between the luminal and abluminal membranes (51, 52). Because of this, endothelial cells are divided into luminal and abluminal domains; different populations of both lipids and proteins exist on each side (2, 3, 48).

O’Kane, Robyn L., and Richard A. Hawkins. 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. First published August 21, 2003; 10.1152/ajpendo.00193.2003.—Several Na⁺-dependent carriers of amino acids exist on the abluminal membrane of the blood-brain barrier (BBB). These Na⁺-dependent carriers are in a position to transfer amino acids from the extracellular fluid of brain to the endothelial cells and thence to the circulation. To date, carriers have been found that may remove nonessential, nitrogen-rich, or acidic (excitatory) amino acids, all of which may be detrimental to brain function. We describe here Na⁺-dependent transport of large neutral amino acids across the abluminal membrane of the BBB that cannot be ascribed to currently known systems. Fresh brains, from cows killed for food, were used. Microvessels were isolated, and contaminating fragments of basement membranes, astrocyte fragments, and pericytes were removed. Abluminal-enriched membrane fractions from these microvessels were prepared. Transport was Na⁺ dependent, voltage sensitive, and inhibited by 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid, a particular inhibitor of the facilitative large neutral amino acid transporter 1 (LAT1) system. The carrier has a high affinity for leucine (Kₘ 21 ± 7 μM) and is inhibited by other neutral amino acids, including glutamine, histidine, methionine, phenylalanine, serine, threonine, tryptophan, and tyrosine. Other established neutral amino acids may enter the brain by way of LAT1-type facilitative transport. The presence of a Na⁺-dependent carrier on the abluminal membrane capable of removing large neutral amino acids, most of which are essential, from brain indicates a more complex situation that has implications for the control of essential amino acid content of brain.

Active transport; brain extracellular fluid; capillaries; endothelial cells; essential amino acids

Nutrients and other molecules must therefore pass two sheaths of membrane. It is the combined characteristics of these membranes that determine which molecules traverse the BBB and how quickly.

Early studies of transport in vivo revealed a distinct pattern of neutral amino acid uptake by brain; movement of essential neutral amino acids from blood to brain was greater than that of nonessential amino acids (1, 32); the latter were immaterial (33). A range of naturally occurring large neutral amino acids (asparagine, cysteine, glutamine, histidine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine) share a single transport system (33). This carrier is facilitative and Na⁺ independent, and L-amino acids are preferred (33); it seems to belong to the L system (leucine preferring), originally described by Oxender and Christensen (34). The carrier is most probably the high-affinity form (3, 45, 46), currently referred to as large neutral amino acid transporter 1 (LAT1) (4, 24, 25, 43). Fernstrom and Wurtman (16) demonstrated the important role of the LAT1 system. They showed that brain tryptophan and serotonin contents and serotonin concentrations were correlated with the ratio of tryptophan to neutral amino acids that exists in plasma. They concluded that competition between tryptophan and other large neutral amino acids for entry to brain was important.

Although Na⁺-dependent systems do not seem to exist at the luminal side (46), several have been found at the abluminal membrane by use of techniques in vitro. System A (alanine preferring) was first characterized and shown to actively transport small nonessential neutral amino acids (3). At least four other Na⁺-dependent carriers exist at the abluminal membrane: system ASC [alanine, serine, and cysteine preferring (17, 47, 50)], system B(−) [basic amino acids, e.g., lysine (30, 41)], system N [e.g., glutamine, asparagine, and histidine (27)], and excitatory amino acid carriers (EAAC) such as aspartate and glutamate (22, 31)]. These Na⁺-dependent carriers provide mechanisms for exporting nonessential amino acids that may be neurotransmitters, nitrogen-rich amino acids, and acidic (excitatory) amino acids that may be detrimental to brain function if they were allowed to accumulate in...
the extracellular fluid (44, 49). Thus the concept arose of the BBB being two complementary membranes working in concert to eliminate noxious molecules (18).

Recently, Na⁺-dependent transport of phenylalanine, an essential aromatic amino acid, was detected at the abluminal membrane (41). The purpose of the present experiments was to study the Na⁺-dependent phenylalanine carrier more closely. The results indicate a Na⁺-dependent carrier on the abluminal membrane of the BBB that preferentially transports aromatic and branched-chain amino acids and is in a position to remove these from the extracellular fluid (ECF) of brain.

MATERIALS AND METHODS

Materials. L-[14C]leucine (292 mCi/mmol) and L-[4,5-3H]-isoleucine (40 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO), amino acids, collagennase type IA, α-(methylamino)isobutyric acid (MeAIB), and 2-aminoacyclohexan-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 Packing (North Aurora, IL). The cows were killed for food under US Department of Agriculture supervision, and the meat was sold for human consumption.

Isolation and characterization of membrane vesicles. Membrane vesicles from brain endothelial cells were prepared as previously described (40). Briefly, isolated microvesicles from bovine cerebral cortexes were observed as described by Partridge et al. (37). The microvesicles were digested with collagenase type IA to remove the basement membrane, pericytes, and glial fragments. The refined microvesicles were homogenized to release the membranes, which were then separated into five fractions at the interfaces of a discontinuous Ficoll gradient (0, 5, 10, 15, and 20%) (40). The amount of abluminal and luminal membrane in each fraction was determined by the activities of two markers previously demonstrated to be located exclusively on one of the membranes. System A transport of MeAIB was used as the marker of the abluminal membrane and γ-glutamyl transpeptidase as the marker of the luminal membrane (40). The typical percentages of luminal membrane in each fraction were F1, 83%; F2, 47%; F3, 34%; F4, 24%; and F5, 34%. Fractions F3 and F4 were combined, resulting in a preparation with an abluminal membrane content of 70%. The contamination by luminal membrane in each fraction were F1, 83%; F2, 47%; F3, 34%; F4, 24%; and F5, 34%. Fractions F3 and F4 were combined, resulting in a preparation with an abluminal membrane content of 70%. The contamination by luminal membrane in each fraction were F1, 83%; F2, 47%; F3, 34%; F4, 24%; and F5, 34%.
System ASC and aromatic amino acid transport. The presence of System ASC (which has a preference for alanine, serine, and cysteine) on the BBB has been suggested (17, 47), and we confirmed its activity in the BBB (30). To determine whether the ASC system could have been responsible for the Na⁺-dependent phenylalanine transport observed by Sánchez del Pino et al. (41), [³H]alanine (0.4 μM) transport was measured in the presence of three aromatic amino acids (phenylalanine, tryptophan, and tyrosine). No significant inhibition was observed, indicating that System ASC could not explain the aromatic amino acid transport (Fig. 2).

System γ-L is a broad-scope amino acid transporter that was first identified in human erythrocytes (11, 12, 35). System γ-L has two distinctive properties: it can bind and translocate cationic and neutral amino acids, and its specificity varies depending on the ionic composition of the medium. In a Na⁺ medium, it transports a variety of neutral amino acids, with a particular affinity for leucine, which binds most strongly. We measured the Na⁺-dependent transport of [¹⁴C]leucine in the presence of a range of neutral, basic, and acidic amino acids (Table 1). Eight large neutral amino acids and two small (alanine and glycine) significantly inhibited Na⁺-dependent leucine transport and may be considered putative substrates. However, neither lysine nor arginine, both transported by System y⁺L with affinities in the low micromolar range (11, 12, 35), had detectable effects. Therefore, participation by System y⁺L in the Na⁺-dependent transport of phenylalanine or leucine is dubious.

Na⁺ dependence of leucine and isoleucine transport. Because Systems B⁺, ASC, and y⁺L were considered unlikely candidates for Na⁺-dependent transport of phenylalanine and leucine, and the other known Na⁺-dependent systems, A, N, and the various excitatory amino acid transporters (EAATs) have different substrate preferences (they do not transport large neutral essential amino acids), we conducted additional experiments. It seemed possible that an as-yet- unidentified system may be present and active on the BBB. One possibility was a Na⁺-dependent system for the branched-chain amino acids leucine, isoleucine, and valine similar to that described by Jørgensen et al. (23).

If a process is Na⁺ dependent, the concentration of substrate within vesicles may transiently exceed the equilibrium value in the presence of an inwardly directed Na⁺ gradient, a so-called “overshoot” (19). Therefore, the demonstration of an overshoot is evidence of a Na⁺-dependent process. The transport of

![Graph](image1)

**Fig. 1.** Lysine transport is not inhibited by 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH). [¹⁴C]lysine (80 μM) transport was measured in the presence of an inwardly directed Na⁺ gradient (100 mM external, internal, nil). BCH did not inhibit at any of the indicated concentrations. Each observation was expressed as the mean of 3 determinations ± SE.

![Graph](image2)

**Fig. 2.** Na⁺-dependent alanine transport in the presence of aromatic amino acids. Net [³H]alanine (0.4 μM) transport was measured in the presence of an inwardly directed Na⁺ gradient (100 mM external, internal, nil). Measurements were corrected for influx measured in the absence of a Na⁺ gradient. α-(Methylamino)isobutyric acid (MeAIB, 20 mM) was included to block System A (alanine preferring). Three aromatic amino acids, 2 mM phenylalanine, 2 mM tryptophan, and 2 mM tyrosine, were tested. No significant inhibition was observed. Each observation was expressed as the mean of 3 determinations ± SE.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Normalized Velocity</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>37.2 ± 3.2</td>
<td>62.9*</td>
</tr>
<tr>
<td>Valine</td>
<td>41.5 ± 3.1</td>
<td>58.5*</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>45.0 ± 3.3</td>
<td>55.0*</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>49.7 ± 5.1</td>
<td>50.3*</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>50.5 ± 4.7</td>
<td>49.5*</td>
</tr>
<tr>
<td>Methionine</td>
<td>58.9 ± 2.5</td>
<td>41.1*</td>
</tr>
<tr>
<td>Alanine</td>
<td>65.1 ± 4.1</td>
<td>34.9*</td>
</tr>
<tr>
<td>Histidine</td>
<td>72.1 ± 14.1</td>
<td>27.9*</td>
</tr>
<tr>
<td>Threonine</td>
<td>75.2 ± 6.2</td>
<td>24.8*</td>
</tr>
<tr>
<td>Glycine</td>
<td>77.6 ± 10.5</td>
<td>22.4*</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>80.8 ± 5.9</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>80.9 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>81.0 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>83.2 ± 11.1</td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>85.5 ± 7.1</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>86.4 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>91.7 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>93.9 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>96.5 ± 14.9</td>
<td></td>
</tr>
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</table>

All values are expressed as the mean ± SE of 3 determinations. Membrane vesicles were incubated with 80 μM [¹⁴C]leucine. Inhibition of Na⁺-dependent leucine transport by various neutral amino acids (2 mM) was determined. Velocity values were corrected for influx measured in the absence of a Na⁺ gradient and normalized to the control. *Statistically significant inhibition (P < 0.05). Statistical analyses were performed using ANOVA and Fisher’s least significant difference test for multiple comparisons.
tracer concentrations of [14C]leucine and [3H]isoleucine was measured in the presence of initial external concentrations of 100 mM Na⁺ or 100 mM choline (Fig. 3). The concentrations of both branched-chain amino acids were substantially increased by the inwardly directed Na⁺ gradient, and an overshoot, indicating a Na⁺-driven process, was observed for both amino acids.

**Effect of transmembrane potential on Na⁺-dependent leucine transport.** The initial rate of Na⁺-dependent [14C]leucine (33 μM) was measured over a range of voltage from +10.7 to −80.7 mV (Fig. 4). The data indicated voltage sensitivity and suggested that a positive charge was translocated with leucine.

**Inhibition of isoleucine transport.** Isoleucine transport was measured in the presence of other branched-chain amino acids (valine and leucine), MeAIB, a specific substrate of System A, and BCH, a substrate of System L (Fig. 5). Valine, leucine, and BCH inhibited isoleucine transport nearly completely, suggesting a carrier that recognizes branched-chain amino acids. MeAIB did not inhibit, providing further evidence that System A, which is inhibited by MeAIB, was not involved.

**Kinetics of leucine transport.** The initial rate of Na⁺-dependent leucine transport (10 s) was measured over a range of leucine concentrations up to 1 mM. The data showed the apparent $K_m$ to be 21 ± 7 μM with a $V_{max}$ value of 114 ± 6 pmol·mg⁻¹·min⁻¹ and a PSA value of 5.4 μl·mg⁻¹·min⁻¹ (Fig. 6). The apparent $K_m$ was similar to that found by Smith et al. (32) for the facilitative carrier (Na⁺ independent) of the BBB.

**Concluding comments.** Amino acids and other nutrients may enter brain through the endothelial cells making up the BBB, the choroid plexus, or the circumventricular organs that have fenestrated capillaries, the last being minute and inconsequential with regard to cerebral nutrition. Pardridge (36) noted that the BBB has a surface area >1,000 times that of the choroid plexus, which forms the blood-cerebrospinal fluid barrier. The large BBB surface area means that the extent to which a given molecule enters brain is determined almost exclusively by the permeability characteristics of the BBB (36).
Oldendorf and Szabo (33) demonstrated that three facilitative amino acid transport systems exist in the BBB: one for basic amino acids, one for large neutral amino acids, and one for acidic amino acids. For many years it was thought that the content of amino acids in brain was controlled by these facilitative systems. But with the ability to examine the membranes of the BBB separately, it has become clear that the situation is far more complex.

The concentrations of all amino acids, with the exception of glutamine, are 6–30 times less in the ECF of brain than in plasma (Table 2). On the other hand, arteriovenous differences of most amino acids are very small or undetectable (13, 15, 39). In other words, amino acids are leaving the brain against a concentration gradient at about the same rate at which they enter. The efflux of amino acids similar to influx cannot be explained by facilitative transport systems: energy must be involved. It must be concluded that the active (e.g., Na⁺-dependent) systems on the abluminal membrane have an important role in maintaining both homeostasis of brain amino acid content and the lower concentration in the ECF. On the basis of similar observations, Bradbury (5) wrote, “[t]here is a strong indirect argument in favor of the hypothesis that most amino acids must be moved against a concentration gradient from interstitial fluid to blood.”

Several Na⁺-dependent carriers are now known to be located in the abluminal membranes of the BBB in a position to export most naturally occurring amino acids, thereby explaining the significantly lower concentrations of amino acids in the ECF of brain. These systems include A (3), ASC (17, 30, 47), N (27), EAAT1–3 (31), and the large neutral amino acids described herein.

The present results establish that Na⁺-dependent transport of large neutral amino acids exists in the abluminal membrane of the BBB. The spectrum of

Table 2. Amino acid concentrations in plasma and brain

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>MW</th>
<th>Nutritional Status in Brain</th>
<th>Plasma Conc</th>
<th>CSF Conc</th>
<th>Plasma/CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>75</td>
<td>NE</td>
<td>139</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>Alanine</td>
<td>89</td>
<td>NE</td>
<td>237</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>Serine</td>
<td>105</td>
<td>NE</td>
<td>86</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>Proline</td>
<td>115</td>
<td>NE</td>
<td>139</td>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>Threonine</td>
<td>119</td>
<td>E</td>
<td>77</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Cysteine</td>
<td>121</td>
<td>E</td>
<td>64*</td>
<td>3*</td>
<td>21</td>
</tr>
<tr>
<td>Methionine</td>
<td>149</td>
<td>E</td>
<td>20</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Asparagine</td>
<td>132</td>
<td>NE</td>
<td>53</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Glutamine</td>
<td>146</td>
<td>NE</td>
<td>669*</td>
<td>547*</td>
<td>1</td>
</tr>
<tr>
<td>Histidine</td>
<td>155</td>
<td>NE</td>
<td>40</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Valine</td>
<td>117</td>
<td>E</td>
<td>174</td>
<td>12</td>
<td>15</td>
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<tr>
<td>Leucine</td>
<td>131</td>
<td>E</td>
<td>71</td>
<td>6</td>
<td>12</td>
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<tr>
<td>Isoleucine</td>
<td>131</td>
<td>E</td>
<td>46</td>
<td>3</td>
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<tr>
<td>Phenylalanine</td>
<td>165</td>
<td>E</td>
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<td>5</td>
<td>9</td>
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<tr>
<td>Tyrosine</td>
<td>181</td>
<td>E</td>
<td>61</td>
<td>7</td>
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<tr>
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<td>204</td>
<td>E</td>
<td>30</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Lysine</td>
<td>146</td>
<td>E</td>
<td>147</td>
<td>9</td>
<td>16</td>
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<tr>
<td>Arginine</td>
<td>174</td>
<td>E</td>
<td>54</td>
<td>10</td>
<td>5</td>
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<tr>
<td>Ornithine</td>
<td>169</td>
<td>NE</td>
<td>44</td>
<td>2</td>
<td>22</td>
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<tr>
<td>Aspartic Acid</td>
<td>133</td>
<td>NE</td>
<td>4</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>147</td>
<td>NE</td>
<td>90</td>
<td>3</td>
<td>30</td>
</tr>
</tbody>
</table>

Concentration values (μM) are from Ref. 28, except where noted as * (26). Cerebral spinal fluid concentrations (CSF Conc) are assumed to approximate brain extracellular fluid (ECF) concentrations (10). MW, molecular weight; E, essential; NE, nonessential. As mentioned, with the exception of glutamine, the concentration of amino acids in the ECF is much lower than in plasma.
inhibitors is similar to the substrates of the facilitative system LAT1 (Table 1). BCH, as well as a wide range of amino acids that may be considered putative substrates, inhibits this Na\(^+\)-dependent system. It seems likely, on the basis of the spectrum of substrates, that the Na\(^+\)-dependent carrier on the abluminal membrane is similar to carriers reported in rabbit kidney (23) and bacteria (21). The system also has some similarities to the ASC/β-pathway described by Pollard et al. (38), but it is different in that alanine (a substrate of ASC/β) transport is not inhibited by phenylalanine, tryptophan, or tyrosine in the BBB Na\(^+\)-dependent system.

Although the BBB determines the availability, and thereby the brain content, of essential amino acids, astrocytes and neurons participate in maintaining the extracellular concentrations. Astrocytes and neurons have Na\(^+\)-dependent transport systems capable of transporting neutral and acidic amino acids (8, 9, 29, 42). These various systems are actively involved in regulating the concentrations in ECF and are especially important in the maintenance of low concentrations of neurotransmitter amino acids such as glutamate, aspartate, and glycine. On the other hand, it now seems clear that the BBB also participates in the active regulation of brain ECF and that the abluminal membrane is especially important in this role.

The emerging view is that cerebral endothelial cells participate actively in regulating the composition of brain ECF and the amino acid content of brain. The two membranes seem to be working in a complementary fashion, with Na\(^+\)-dependent transport of amino acids occurring at the abluminal membrane and facilitative transport at the luminal or, in the case of large neutral amino acids, at both membranes (18).

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**DISCUSSIONS**

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