Branched-Chain Amino Acids: Metabolism, Physiological Function, and Application

Structure of the Blood–Brain Barrier and Its Role in the Transport of Amino Acids[1–3]

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ABSTRACT Brain capillary endothelial cells form the blood–brain barrier (BBB). They are connected by extensive tight junctions, and are polarized into luminal (blood-facing) and abluminal (brain-facing) plasma membrane domains. The polar distribution of transport proteins mediates amino acid (AA) homeostasis in the brain. The existence of two facilitative transporters for neutral amino acids (NAAs) on both membranes provides the brain access to essential AAs. Four Na^+-dependent transporters of NAA exist in the abluminal membranes of the BBB. Together these systems have the capability to actively transfer every naturally occurring NAA from the extracellular fluid (ECF) to endothelial cells and from there into circulation. The presence of Na^+-dependent carriers on the abluminal membrane provides a mechanism by which NAA concentrations in the ECF of brain are maintained at ~10% those of the plasma. Also present on the abluminal membrane are at least three Na^+-dependent systems transporting acidic AAs (EAAT) and a Na^+-dependent system transporting glutamine (N). Facilitative carriers for glutamine and glutamate are found only in the luminal membrane of the BBB. This organization promotes the net removal of acidic- and nitrogen-rich AAs from the brain and accounts for the low level of glutamate penetration into the central nervous system. The presence of a γ-glutamyl cycle at the luminal membrane and Na^+-dependent AA transporters at the abluminal membrane may serve to modulate movement of AAs from blood to the brain. The γ-glutamyl cycle is expected to generate pyroglutamate (synonymous with oxyproline) within the endothelial cells. Pyroglutamate stimulates secondary active AA transporters at the abluminal membrane, thereby reducing the net influx of AAs to the brain. It is now clear that BBB participates in the active regulation of the AA content of the brain. J. Nutr. 136: 218S–226S, 2006.

KEY WORDS: • *brain capillaries* • *endothelial cells* • *polarity* • *sodium dependent transport* • *facilitative transport*

The brain is sheltered from the changing metabolite concentrations in blood by a blood–brain barrier (BBB)[5] that surrounds the central nervous system (CNS) including the spinal cord (**Fig. 1**). The BBB is necessary to provide an optimal chemical environment for cerebral function. Several layers exist between blood and brain: capillary endothelial cells, a basement membrane consisting of type IV collagen; fibronectin and

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[5] Abbreviations used: AA, amino acid; BBB, blood–brain barrier; BCH, 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid; CNS, central nervous system; CSF, cerebrospinal fluid; ECF, extracellular fluid; GGT, γ-Glutamyl transpeptidase; LNAA, large neutral amino acid; MeAIB, *N*-(methylamino)-isobutyric acid; NAA, neutral amino acid; NH_4^+, ammonia (because this is the predominant form at pH 7.4). The following abbreviations are used for Na^+-dependent transport systems: A, (alanine-preferring (carries small NAA); ASC, alanine-, serine-, cysteine-preferring; N, glutamine-preferring (1,2); EAAT, excitatory amino acid transporter [a family of glutamate-aspartate transporters (K^+ antiporter)] (3-5). The Na^+-LNAA transport of large neutral amino acids was recently described functionally (6). The following abbreviations are used for facilitative transport systems: L1, leucine-preferring (carries LNAA) (7); n, glutamine-preferring (2); y^+, cationic-AA transporter; x_G^-, acidic AA transporter (glutamate- and aspartate-preferring) (8); x_C^-, electro-neutral glutamate/cystine exchanger (4,5).

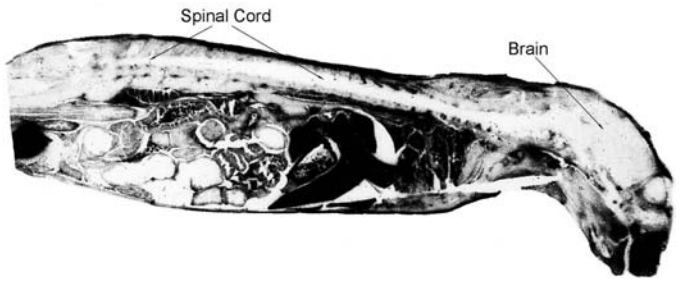

FIGURE 1 The blood–brain barrier extends throughout the central nervous system. Saggital section through a mouse showing the distribution pattern of I^{131} labeled Renografin™, an hydrophilic dye that does not pass the blood–brain barrier, 15 min after injection. All tissues take up the dye except the entire central nervous system (101). Photo courtesy of Professor V. Nair, Rosalind Franklin University of Medicine and Science.

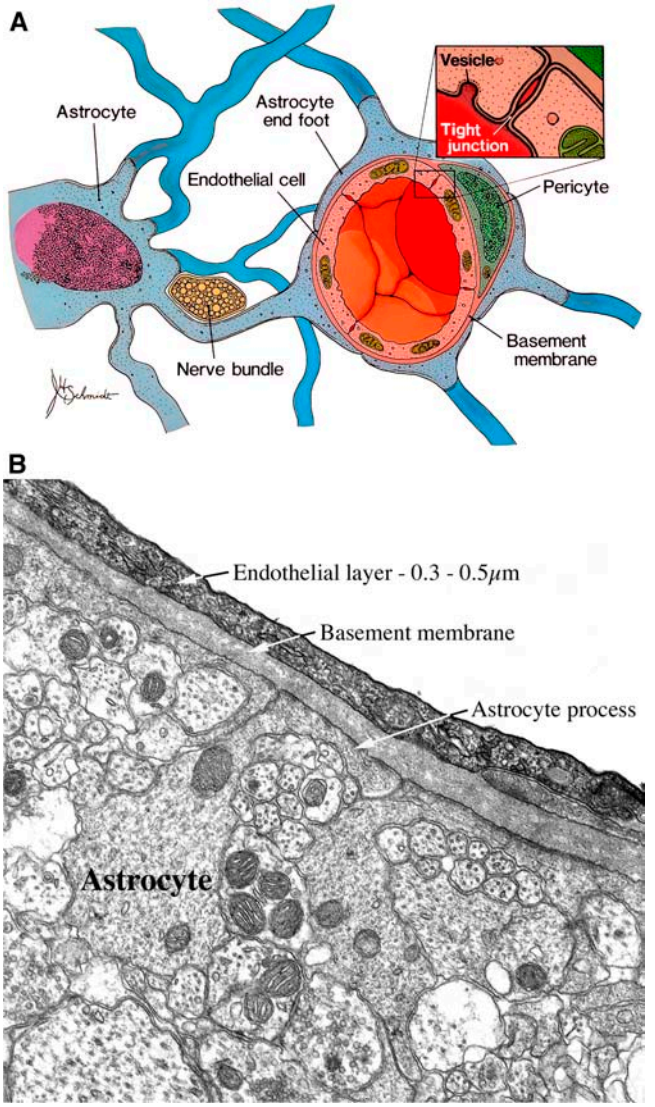

FIGURE 2 (A) The BBB exists at the level of the endothelial cells of cerebral capillaries. The endothelial cells are joined together by an extensive network of tight junctions and surrounded by a basement membrane, within which pericytes reside. Astrocytic processes (so-called end-feet) surround cerebral capillaries (previously published in IUBMB Life). (B) Right, an electron micrograph of a cerebral capillary shows the basic elements. The electron micrograph was provided through the courtesy of Robert Page, MD; Professor, Neurosurgery and Anatomy, Pennsylvania State University College of Medicine.

laminin that completely covers the capillaries; pericytes embedded in the basement membrane; and astrocyte processes that surround the basement membrane. Each of these layers could, potentially, restrict the movement of solutes (**Fig. 2**).

Endothelial cells were demonstrated to be the site of the BBB when it was observed that horseradish peroxidase could not pass the endothelial layer from either direction (9–11). Although Pappenheimer (12) challenged this concept, arguing that astrocytes were a more likely site of the barrier, Crone (13) definitely decided the issue by demonstrating that brain capillaries from amphibians, which have no surrounding layer of astrocytes, have high electrical impedance, $\approx$ 2,000 $\Omega \times cm^2$, which is indicative of a restriction to the movement of ions. The cerebral endothelium is now accepted as the site of the BBB in higher animals (13).

Cerebral capillary endothelial cells differ from other mammalian capillary endothelial cells; they have few cytoplasmic vesicles, more mitochondria (14), and a larger number of tight junctions between overlapping cells. The tight junctions inhibit paracellular movement, prevent membrane molecules from moving from one cell to another (15), and divide the membranes of the endothelial cells into two distinct sides, luminal (blood side) and abluminal (brain side) (16). Different populations of both lipids and intrinsic proteins (e.g., transporters) exist on the luminal and abluminal sides (17–19). Therefore, hydrophilic nutrients must pass two sheaths of membrane, the combined characteristics of which determine which particles traverse the barrier and how quickly. Pappenheimer and Setchell (20) recognized the implication of passing two membranes in series to gain entry to the CNS, and Oyler et al. (21) demonstrated the effect of passing two membranes in series by computer simulation.

Various methods are used to study the transport of solutes across the BBB in vivo and in vitro including single-pass indicator diffusion (22,23), the brain-uptake index (24), in situ brain perfusion (25), isolated brain microvessels (26,27), and cultured endothelial cells (28–30). These techniques give valuable information about transport, but do not distinguish between the different functions of the luminal membranes and abluminal membranes.

Betz et al. (18) developed a procedure to separate the respective plasma membrane domains and convincingly demonstrated a polarity between the two sides. On isolation, luminal and abluminal membranes form sealed spherical vesicles that are predominantly right-side-out and are suitable for the study of transport in vitro (31,32). The isolated membranes maintain functional transport properties and thus may be used to characterize the contribution of each membrane domain to BBB activity.

Until recently the BBB, at least with regard to metabolites, had been viewed as a passive system. The various facilitative transporters were considered to play a role in the regulation of brain metabolism through their ability to limit access (33). In contrast, it was known that active transport of ions existed. Bicarbonate and other ions are actively secreted across the BBB (34,35). (Na^+/K^+)-ATPase is present in the abluminal membrane (36). One of the most important functions of (Na^+/K^+)-ATPase is to maintain the high concentration gradient of Na^+ (external > internal) so Na^+-dependent transport can occur. Furthermore, cerebral endothelial cells have a high density of mitochondria compared with other endothelial cells and, therefore, the capacity for greater energy production (14).

METHODOLOGY

This article focuses on new knowledge concerning amino acid (AA) transport, which was gained by making measurements in separated luminal and abluminal membranes, as described by Sánchez del Pino et al. (32). These membranes form spheres that can be used to measure the uptake of substrates in vitro under controlled conditions (31,32,37). The following sections illustrate that the BBB is an active, not passive, participant in the regulation of the brain's content.

Facilitative amino acid transporters of the BBB

Early studies of AA transport in vivo identified facilitative transporters on the luminal membrane that were saturable and stereoselective (38,39). Luminal carriers of AAs have no dependence on Na^+ gradients (7,39–43). Three broad classes of facilitative carriers exist: large neutral amino acids (LNAAs), cationic AAs, and acidic AAs (44). Currently, four facilitative carriers have been identified L1, y^+, x_G^- and n. L1 and y^+ are present in both membranes (37,45), whereas x_G^- and n are restricted to the luminal membrane (2).

Facilitative transport of large essential neutral amino acids: system L1. Early studies of transport in vitro revealed a distinct pattern of LNAA uptake by brain: movement of essential neutral amino acids (NAAs) from blood to brain was greater than nonessential NAAs (39,40); the movements of the latter were minimal (44). Transport was facilitative, Na^+-independent and NAAs were preferred (44). Therefore, the carrier seems to belong to the L-system (leucine preferring) originally described by Oxender and Christensen (46), and it is probably the high affinity form currently referred to as L1 (7,47–49). Measurements in membranes indicate L1 is present in both membranes in a 2:1 ratio (luminal:abluminal) (37,45). The substrates carried by L1 include leucine, valine, methionine, histidine, isoleucine, tyrosine, tryptophan, phenylalanine, and threonine, most of which are essential. The affinity constants (K_m) are in the μmol/L range and similar to the plasma concentrations (7). Glutamine has also been described as a substrate of L1, but glutamine transport is not completely inhibited by 2-aminobicyclo (2,2,1)-heptane–2-carboxylic acid (BCH), a specific inhibitor of the L1 system. Therefore it seems likely that glutamine transport is also transported by system n (2 and see below).

L1 is undoubtedly the most important source by which essential NAAs gain access to the brain. Fernstrom and Wurtman (50) demonstrated the important role of the L1-system and the competition among LNAAs by showing that brain tryptophan and serotonin contents were correlated with the ratio of tryptophan:LNAA that exist in plasma. They concluded that competition between tryptophan and other LNAAs for entry to the brain is an important factor in determining the content of serotonin in the brain.

Facilitative transport of cationic amino acids: system y^+. System y^+ is the primary cationic AA transporter of the BBB (51). This transporter has an affinity for AAs with cationic side chains, including lysine, arginine, ornithine, and homoarginine in the μmol/L range. In addition, this system exhibits a weak interaction with NAAs if Na^+ is present, and therefore it is referred to as y^+ (5,52). System y^+ is inhibited by many NAAs, but the affinity constants are about 10-fold greater than those of system L1 (45). System L1 must be considered the provider of essential NAAs, whereas y^+ is primarily a purveyor of lysine, arginine, and ornithine. System y^+ exists in both membranes, but predominates on the abluminal side (45). The ability of system y^+ to transport various LNAAs may explain the apparently first-order component often observed in studies of AA transport.

Facilitative transport of glutamine: system n. Lee et al. (2) described facilitative transport of glutamine across the luminal membrane of the BBB that was not inhibited by BCH and did not demonstrate trans-stimulation. This transport system is similar to system n described in hepatic plasma membrane vesicles (53). The BBB system n is inhibited by asparagine and histidine (45) in hepatic vesicles, as found by Pacitti et al. (53). System n exists solely on the luminal membrane (2).

Facilitative transport of acidic amino acids: system x_G^-. Benrabh and Lefauconnier studied glutamate uptake in vitro and found no evidence of Na^+-dependent transport when glutamate was presented to the luminal membrane (8). They concluded the carrier was facilitative and probably the x_G^- form because no evidence for the cystine-glutamate exchanger x_C^- could be found. We confirmed this in isolated luminal membranes. Cystine did not compete with glutamate for uptake, whereas aspartate did. Furthermore, cystine did not accelerate glutamate uptake in vesicles preloaded with 2 mmol/L cystine (R. A. Hawkins, A. Mokashi, and I. A. Simpson, unpublished data).

Lee et al. (2) measured facilitative glutamate transport in both luminal and abluminal membranes and found facilitative glutamate transport only on the luminal border in a position to allow the release of glutamate from endothelial cells to the plasma.

A compilation of the substrates carried by the various facilitative systems is presented in **Table 1** and their kinetic characteristics in **Table 2**. The organization of the transporters is depicted in **Figure 3**.

TABLE 1

Amino acids transported by facilitative transport systems

System	L1	y^+	n	x_G^-
Nonessential				
Glycine		*		
Alanine		*		
Serine		*		
Proline				
Asparagine	+		+	
Glutamine	+	*	+	
Aspartate				+
Glutamate				+
Arginine		+		
Ornithine		+		
Essential in brain				
Lysine		+		
Histidine	+	*	+	
Threonine	+	*		
Cysteine		*		
Methionine	+	*		
Valine	+	*		
Leucine	+			
Isoleucine	+			
Phenylalanine	+	*		
Tyrosine	+			
Tryptophan	+			

AAs transported, or shown to inhibit transport, are indicated by a +. Facilitative transport (weak) of NAAs by y^+ in the presence of Na^+ are indicated by *. Systems L1 and y^+ exist on both membranes whereas systems x_G^- and n are restricted to the luminal membrane (2,45). AAs in italics are essential in brain (54). The distribution of these transporters is depicted in Figure 3.

TABLE 2

Kinetic characteristics of facilitative amino acid transporters on the blood–brain barrier

Transporter (Substrate)	Apparent K_m	Apparent V_{max}	Clearance	Position
	mmol/L	*pmol mg^{-1} min^{-1}*	*μl mg^{-1} min^{-1}*	
L1 (Phe)	0.012 ± 0.02	94 ± 9	8	Luminal and abluminal
y^+ (Lys)	0.8 ± 0.3	5,800 ± 1600	7	Luminal and abluminal
n (Gln)	1 ± 0.5	1,100 ± 230	1	Luminal
x_G^- (Glu)	0.9 ± 0.9	700 ± 300	1	Luminal

The radiolabeled substrate used for measurements are in parenthesis. Clearance was calculated to the nearest integer as $V_{max} \div K_m$. Values were taken from (32,45).

Amino acid gradients between brain and plasma

The concentrations of all naturally occurring AAs in the cerebral spinal fluid (CSF) (presumably similar to the extracellular fluid (ECF) of the brain), with the exception of glutamine, are ~10% or less than the plasma concentrations (**Fig. 4**) (54). This situation cannot be explained by the consumption of AAs by brain because the arteriovenous differences across brain of most AAs are imperceptible (55–57), as are the arteriovenous differences of ammonia (NH_4^+), a byproduct of AA catabolism (58). These observations indicate that AAs leave the brain against a concentration gradient. From this it may be concluded that active (e.g., Na^+-dependent) systems on the abluminal membrane have an important role in maintaining both homeostasis of brain AA content as well as the lower concentration in the extracellular fluid. Based on similar observations Bradbury wrote "there is a strong indirect argument in favor of the hypothesis that most AA must be moved against a concentration gradient from interstitial fluid to blood" (34).

Na^+-dependent transport systems of the BBB

The Na^+-dependent systems that have been identified to date include the following: A (alanine preferring), which was first characterized and shown to actively transport small nonessential NAAs (17,32), ASC (alanine-, serine-, and cysteine-preferring) (59–61), N (e.g., glutamine-, asparagine- and histidine-preferring) (2), the excitatory acidic acid amino acid transporter (EAAT) family (e.g., aspartate- and glutamate-preferring) (3,62), and a recently described system that transports primarily essential, LNAAs (6). The latter system has not been named and is referred to as Na^+-LNAA.

Na^+-dependent transport of AAs exists only in abluminal membranes. No Na^+-dependency has been detected in luminal membranes, which appear to have only facilitative carriers (7,39,41,42). Therefore, the Na^+-dependent transporters are in a position to remove AAs from the brain utilizing the Na^+-gradient that exists between the ECF and the endothelial cells of brain capillaries comprising the BBB.

Na^+-dependent transport of large neutral amino acids: system Na^+-LNAA. Initial studies by Sánchez del Pino et al. (37) found Na^+-dependent phenylalanine transport that was inhibited by BCH. Studies by Van Winkle et al. (63), had demonstrated system $B^{o,+}$ is a Na^+-dependent carrier that recognizes NAAs and is inhibited by BCH. Because of this characteristic and the observed inhibition, the authors thought carrier System $B^{o,+}$ was likely to be responsible for the transport activity. A characteristic of System $B^{o,+}$ is the ability to transport cationic AAs (63). However, the rate of lysine transport was not inhibited by the presence of concentrations of BCH up to 10 mmol/L, casting doubt on the presence of System $B^{o,+}$ (6). Further investigation led to the discovery of Na^+-LNAA as the carrier responsible for the BCH-inhibited, Na^+-dependent phenylalanine transport and other LNAAs (6).

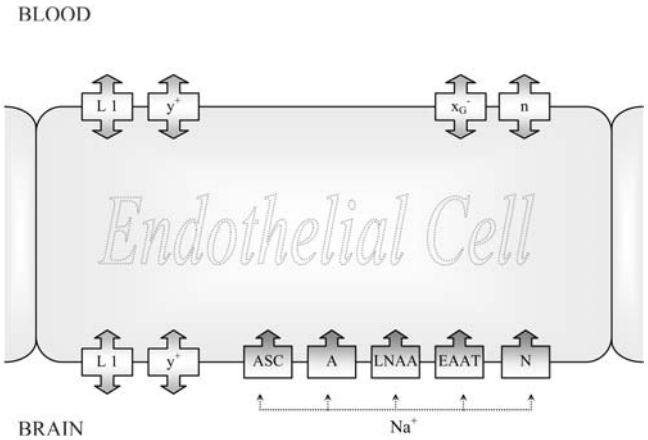

FIGURE 3 Amino acid transporters of the BBB. The brain gains access to all essential AAs through the facilitative systems L1 and y^+ that exist on each membrane. Facilitative transporters x_G^- and n exist only on the luminal membrane and in a position to allow glutamate, aspartate, and glutamine egress. Each facilitative transporter carries several substrates (Table 2). The Na^+-dependent transport systems provide mechanisms for the elimination of nonessential AAs, toxic AAs, and maintain the optimal concentrations of all other AAs. As with the facilitative systems there is considerable overlap of substrates (Table 3). All naturally occurring AAs are transported by at least one system and some by as many as three (Table 3).

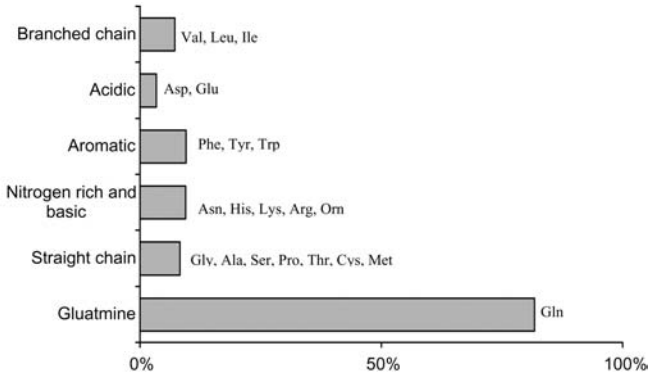

FIGURE 4 Amino acid concentrations in plasma and brain. The plasma and CSF concentrations were grouped and the CSF-to-plasma ratio expressed as percent of the plasma. CSF concentrations are assumed to approximate brain ECF (54,102). With the exception of glutamine, the concentrations of all AAs in the ECF are much lower than the concentrations of AAs in plasma.

Na^+-LNAA was discovered as a distinct transporter in abluminal membrane microvessels and its kinetic characteristics cannot be ascribed to any other currently known systems (6). Na^+-LNAA has a high affinity for leucine (K_m = 21 μmol/L ± 7 SE) and is inhibited by other NAAs including glutamine, histidine, methionine, phenylalanine, serine, threonine, tryptophan, and tyrosine. Transport is Na^+-dependent, voltage sensitive, and inhibited by BCH. The spectrum of AAs carried by Na^+-LNAA is similar to the facilitative system L1 that allows the entry of essential LNAAs down their concentration gradients (compare **Tables 1 and 3**). The presence of a Na^+-dependent carrier on the abluminal membrane, capable of removing LNAAs from the brain, most of which are essential seems to provide a mechanism for the control of the LNAA content of the brain.

Na^+-dependent transport of small nonessential neutral amino acids: system A. The activity of system A, named for its preference for transporting alanine (46) may be distinguished from other Na^+-dependent carriers by its acceptance of *N*-methylamino-isobutyric acid (MeAIB) as a unique substrate (64). System A is voltage sensitive; three positive charges are translocated per MeAIB molecule (1). System A is inhibited by small nonessential AAs such as proline, alanine, histidine, serine, asparagine, and glutamine as well as the essential AA, histidine. Other laboratories (17,46) reported a similar AA spectrum for system A but also included glycine. Glycine transport was not mediated by system A in isolated membrane vesicles but was a putative substrate of system ASC (1).

Na^+-dependent transport of some large and small neutral amino acids: system ASC. ASC activity was measured in abluminal membranes, after blocking system A with MeAIB, confirming the findings of others who have reported its presence (59–61). In addition to alanine, serine, cysteine, and glycine, several essential AAs were putative substrates, including methionine, valine, leucine, isoleucine, and threonine (1). ASC activity is independent of the transmembrane potential (1).

TABLE 3

Amino acids transported by Na^+-dependent systems of the abluminal membrane

System	A	N	ASC	Na^+LNAA	EAAT
Nonessential					
Glycine			+	+	
Alanine	+		+	+	
Serine	+	+	+		
Proline	+				
Asparagine	+	+			
Glutamine	+	+			
Aspartate					+
Glutamate					+
Essential in brain					
Histidine	+	+		+	
Threonine			+	+	
Cysteine			+		
Methionine			+	+	
Valine			+	+	
Leucine			+	+	
Isoleucine			+	+	
Phenylalanine				+	
Tyrosine				+	
Tryptophan				+	

AAs that are transported, or shown to inhibit transport, are indicated by a +. Values for systems A, N, and ASC are from (1). Values for Na^+-LNAA are from (6). Values for the EAAT1–3 family are from (3). AAs in italics are essential in brain (54).

Na^+-dependent transport of nitrogen rich amino acids: system N. System N has a preference for NAAs that are nitrogen rich, such as glutamine, histidine, and asparagine, hence its designation (65,66). BBB abluminal membranes also transport serine via this system. System N was not affected by the transmembrane potential (1). Li^+ could substitute for Na^+, suggesting that system N in the BBB is similar to system N in liver cells (1,65).

Na^+-dependent transport of acidic amino acids: the EAAT family. Na^+-dependent glutamate transporters exist on the abluminal membrane. They are voltage dependent, and collectively have an apparent K_m of 14 μmol/L at a transmembrane potential of –61mV (2,3). Analysis of mRNA demonstrated that 3 transporters were expressed (EAAT1, 2, and 3) in brain capillary endothelial cells. Western blot analysis confirmed the glutamate transporters to be present only on the abluminal membranes; none were detectable on luminal membranes (3). The activity of the 3 transporters was 1:3:6, EAAT1: EAAT2: EAAT3, respectively. Collectively the EAAT family is the most powerful of the Na^+-dependent AA transporters; they show the greatest ability to clear AAs at low concentrations (**Table 4**).

Overview of the organization of the various transport systems

The brain gains access to all essential AAs through the facilitative systems L1 and y^+. There is considerable substrate overlap for within the facilitative systems as well as within the Na^+-dependent systems (Tables 1 and 3).

Five Na^+-dependent AA transport systems are present exclusively on the abluminal membrane of the BBB (Fig. 3) and the capacities of these transporters are similar to or greater than those of the facilitative transporters. Because the electrochemical gradient for Na^+ is oriented to flow from the extracellular fluid into the endothelial cells, these Na^+-dependent transport systems are in a position to export AAs from the brain's extracellular fluid. Thus, AAs that pass both endothelial cell membranes and enter the basement membrane space could be actively, and selectively, pumped back across the abluminal membrane. This asymmetrical distribution of Na^+-dependent carriers has the potential, therefore, to reduce the content of AAs in the brain.

The Na^+-dependent transport systems provide a mechanism for the elimination of nonessential AAs and toxic AAs as well as maintaining the optimal concentrations of all other AAs. As with the facilitative systems, there is considerable substrate overlap. All naturally occurring AAs are transported by at least 1 system and some by as many as 3 (Table 3). The kinetic characteristics are summarized in Table 4. The following sections illustrate how both membranes of the BBB may play an active role in maintaining homeostatic concentrations.

Branched chain amino acids and brain function. It has been suggested that the plasma concentrations of BCAAs may influence brain function and affect appetite (67), physical and mental fatigue (68–70), mental performance (71), physical endurance (72,73), sleep (71), hormonal function, blood pressure, and affective state. (74). Presumably, BCAAs influence brain function by altering the availability of aromatic AAs (50). As mentioned, transport of LNAAs, mediated by the facilitative system L1, is shared by several LNAAs. BCAAs are especially effective in competing with aromatic AAs for entry. Consequently, when plasma BCAA concentrations rise, which can occur in various normal and abnormal situations, they impair the entry of aromatic AAs, notably tryptophan (74). Serotonin synthesis in the brain depends directly on the availability of tryptophan. Therefore, when plasma BCAA

TABLE 4

Kinetic characteristics of Na^+-dependent amino acid transporters in abluminal membranes

Transporter (Substrate)	Apparent K_m	Apparent V_{max}	Clearance	Voltage Sensitivity
	mmol/L	*pmol•mg^{-1}•min^{-1}*	*μl•mg^{-1}•min^{-1}*	
A (MeAIB)	0.4 ± 0.16	500 ± 60	1*	Yes
N (Gln)	1.3 ± 0.4	4,400 ± 700	3	No
ASC (Ala)	0.11 ± 0.06	660 ± 70	6	No
Na^+-LNAA (Leu)	0.021 ± 0.007	114 ± 6	5*	Yes
EAAT (Glu)	0.014 ± 0.004	151 ± 20	11*	Yes

The radiolabeled AAs used for measurements are in parenthesis. Clearance was calculated as V_{max}/K_m. Kinetic values were from: Na^+-LNAA, (6); EAAT1–3, (6); A, ASC and N (1). Values marked by an asterisk were measured at a transmembrane potential of –61 mV. MeAIB (20 mmol/L) was included in measurements of systems N and ASC to exclude transport by system A.

concentrations rise, the contents of brain tryptophan and serotonin fall (74). Although the focus of LNAA transport has been on the facilitative system L1, the recent discoveries that Na^+-dependent carrier systems are present on the abluminal membrane of the BBB (1,6), adds a new element that must be considered. These Na^+-dependent carriers are capable of propelling all NAAs, including BCAAs and aromatic AAs, back toward the plasma. Thus future directions should consider mechanisms that affect the retention of AAs by the brain once they have entered.

Glutamate, glutamine, and ammonia removal. Glutamate is the most abundant AA in the brain and the primary excitatory neurotransmitter of the CNS (75). To maintain the very low concentrations of glutamate in extracellular fluid (1–3 μmol/L) (76,77) [whereas intracellular glutamate is 4000–12,000 times greater (~12,000 μmol/g)] energy-dependent transport is required (75). To date, 5 isoforms of Na^+-dependent glutamate transporters (EAAT) have been described (4). All exist selectively in various neural cells of brain (78–81). Three members of the EAAT family have been shown to be expressed (EAAT1, 2, and 3) in the abluminal membrane of the BBB (3).

The facilitative carrier x_G- is present in the BBB (2,8,45) allowing minimal entry of glutamate to brain (82,83). The brain uptake permeability-surface area product for glutamate from normal plasma is 7 μL min^{-1} g^{-1}, corresponding to an influx rate of only 0.67 nmol × min^{-1} g^{-1}. This influx is only about one-tenth that of most of the LNAAs and cationic AAs (83).

Experiments with enriched fractions of luminal and abluminal vesicles from the BBB confirmed the presence of facilitated transport systems for glutamine (system n) and glutamate (system x_G-), but these carriers exist only in the luminal membrane of the BBB (2). Na^+-dependent glutamine transport occurs in the abluminal membrane, primarily by the N system and to a lesser degree by system A (2). Furthermore, glutaminase exists within the endothelial cells (2).

The glutamate concentration inside endothelial cells increases by 2 main mechanisms: glutamate transport into cells from the ECF, and conversion of glutamine to glutamate by glutaminase. When intracellular glutamate becomes greater than the plasma concentration, net transport of glutamate across the luminal membrane into blood will occur through the facilitative carrier. The absence of facilitative carriers on the abluminal membrane prevents passive movement of glutamate into brain ECF. The presence of Na^+-dependent transporters on the abluminal membrane provides a mechanism to increase intracellular glutamate concentration and therefore the removal of glutamate from the endothelial cells. This mechanism, as well as the roles of astrocytes and neurons is summarized in **Figure 5**. Thus, the 2 membranes of the BBB are organized in a manner that removes glutamine and glutamate from the brain, thereby maintaining homeostasis.

These observations also provide an explanation for a long-standing mystery regarding brain NH_4^+ metabolism. Various measurements have shown that 20–50% of the NH_4^+ circulating through brain blood vessels passes the BBB and is incorporated into the amide group of glutamine by astrocytes (58,84). It is curious, however, that it has not been possible to consistently measure arteriovenous differences of NH_4^+ (58). If there were no mechanism for the removal of glutamine it would

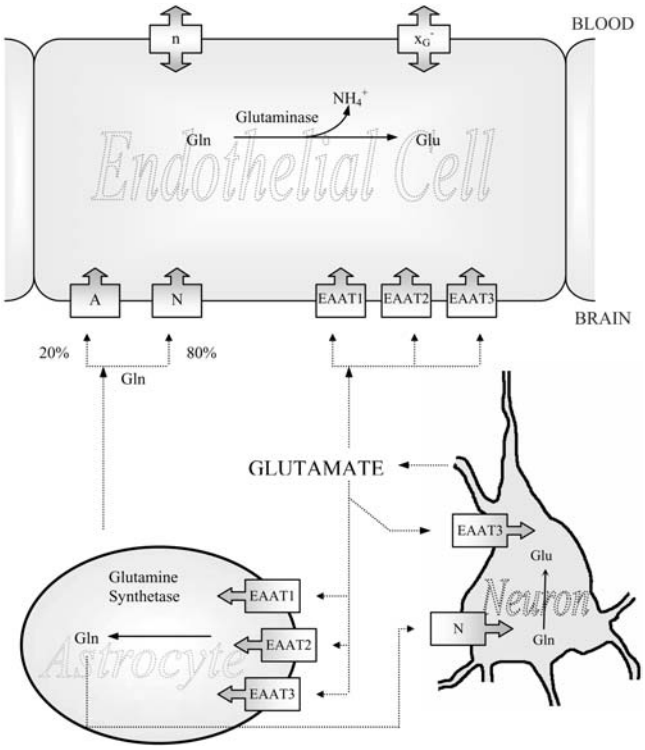

FIGURE 5 Glutamate and glutamine transport between neurons, astrocytes, and endothelial cells. The presence of Na^+-dependent carriers capable of pumping glutamine and glutamate from brain into endothelial cells, glutaminase within endothelial cells to hydrolyze glutamine to glutamate and NH_4^+, and facilitative carriers for glutamine and glutamate at the luminal membrane provides a mechanism for removing nitrogen and nitrogen-rich AAs from brain (2). EAAT1, 2, and 3 are present in endothelial cells (3), and astrocytes, EAAT3 is present in nerve cells (109). A, Na^+-dependent system A; N, Na^+-dependent system N; EAAT, Na^+-dependent glutamate transporter, x_G-, facilitative glutamate transporter, n, facilitative glutamine transporter.

accumulate in the brain, thereby raising osmolarity and causing swelling. The situation is now clarified. Glutamine and glutamate are pumped from ECF into endothelial cells. Glutamine is at least partially metabolized to NH_4^+ and glutamate, and NH_4^+, glutamate, and glutamine are free to diffuse across the luminal membrane into blood (2,3).

This new knowledge also explains how the entry of glutamine and glutamate to the CNS is restricted even though carrier activities for both AAs have been described (44,83,85). Glutamine and glutamate can traverse the luminal membrane on facilitative systems. However, movement into brain, across the abluminal membrane, is small because of a lack of facilitative carriers on the abluminal membrane. Furthermore there are 3 Na^+-dependent carriers on the abluminal membrane that are driven by the steep Na^+ gradient that exists between brain extracellular fluid and the cell interior. These carriers forcefully oppose glutamate entry and promote its removal from the brain.

The BBB seems to be arranged in such a manner as to not only restrict the entry of glutamine and glutamate into brain but also actively export these AAs, and possibly NH_4^+, to the circulation. Therefore, the BBB participates in the regulation of brain nitrogen metabolism, and protects against the development of neurotoxicity by preventing the accumulation of glutamate as well as the accumulation of NH_4^+.

The γ-glutamyl cycle and the role of pyroglutamate on Na^+-dependent carriers. The γ-glutamyl cycle proposed by Meister (86,87) accounts for the synthesis and degradation of reduced glutathione (GSH) and has been shown to influence AA transport in various tissues. The original suggestion that the cycle is involved directly in AA translocation into cells is controversial, having received support and criticism. However, studies using lactating mammary glands and the placenta from pregnant rats showed that pyroglutamate (also known as oxoproline), an intermediate of the γ-glutamyl cycle, serves to stimulate Na^+-dependent AA transport (88,89).

The first reaction of the cycle occurs extracellularly and is catalyzed by γ-glutamyl transpeptidase (GGT) (**Fig. 6**) (90). The substrates for GGT are glutathione, which is exported across the luminal membrane of endothelial cells to the plasma side, and extracellular AAs in the plasma. The γ-glutamyl-AAs that result enter cells by a transport system that is not shared by free AAs. Intracellularly, γ-glutamyl-AAs are substrates of γ-glutamyl cyclotransferase, which converts the γ-glutamyl-AAs into pyroglutamate and the corresponding free AAs. Subsequently pyroglutamate is hydrolyzed to glutamate by oxoprolinase (91).

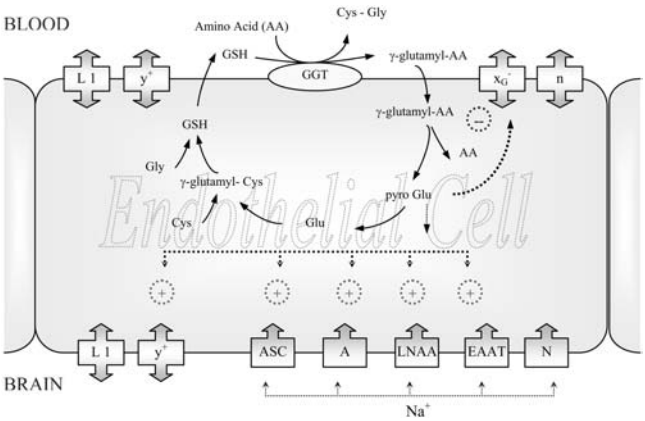

FIGURE 6 The influence of pyroglutamate on AA transport across the blood–brain barrier. γ-Glutamyl-AAs are formed at the outer surface of luminal membranes of the endothelial cells by GGT that transfers the γ-glutamyl moiety of glutathione to most AAs, thereby forming a γ-glutamyl-AA. The γ-glutamyl-AA enters endothelial cells where the AA is released and pyroglutamate is formed. The Na^+-dependent transport systems A, ASC, and Na^+-LNAA, EAAT, and y+, all located on the abluminal side, are activated by pyroglutamate. N was the only system not stimulated. L1 is present on both the luminal and abluminal membrane and is not affected by pyroglutamate (2).

Using abluminal membrane vesicles from the BBB, it was shown that pyroglutamate stimulates the Na^+-dependent system A by 70% in a concentration-dependent manner (92). Thus, the affinity for MeAIB was increased by 50%, with no change in V_{max}. Pyroglutamate had no effect on luminal transport of L-phenylalanine (a representative substrate of the facilitative transport system, L1); the effect of pyroglutamate was restricted to the Na^+-dependent AA transport systems of the abluminal membrane (92).

Recently we studied the effect of pyroglutamate on the other Na^+-dependent transporters of the BBB (R. A. Hawkins and A. Mokashi, unpublished data). We found that preloading membrane vesicles with 2 mmol/L pyroglutamate stimulated all Na^+-dependent AA transport systems with the exception of system N, which transports glutamine. The latter is interesting because glutamine is the only AA present in similar concentrations in plasma and ECF and is synthesized from NH_4^+ that enters brain continuously (58). Also of interest was the finding that pyroglutamate stimulated y^+, a transporter that transports cationic AAs, but also transports a range of NAAs in the presence of Na^+ (5,52).

The presence of GGT in the BBB has been an enigma. GGT activity is high in tissues that actively transport AAs, such as the brush border of the proximal convoluted tubules of the kidney (93), the lactating mammary gland (93), and the apical portion of the intestinal epithelium (93). The BBB differs from these tissues in that it is not associated with active AA uptake from plasma. Although the brain requires essential AAs for its function and growth, their supply is not much greater than the demand, and it is difficult to detect arteriovenous differences of AAs across the brain (55,57). It has, therefore, been puzzling as to why brain capillaries have such high GGT activity.

Our data support the hypothesis that the γ-glutamyl cycle influences AA transport systems indirectly through pyroglutamate, produced intracellularly as an intermediary metabolite of the γ-glutamyl cycle. Pyroglutamate, in turn acts to stimulate Na^+-dependent AA transport systems. The γ-glutamyl cycle and GGT may serve to monitor the availability of AAs to the brain and constitute the first step in a control mechanism that influences the accessibility and content of brain AAs (Fig. 6). The question arises as to whether pyroglutamate concentrations that exist in vitro are sufficient to stimulate Na^+-dependent transport. Although we are unaware of measurements of pyroglutamate in cerebral microvessels, the concentrations in normal human plasma and various tissue extracts are between 20 and 50 μmol/L (91,94), and as high as 6 mmol/L in plasma and cerebrospinal fluid in pathological conditions (95). Stimulation of Na^+-dependent transport of system A was a linear function of the pyroglutamate concentration up to 2 mmol/L, a range that does not seem unreasonable.

The transpeptidation activity of GGT is a function of the plasma concentration and spectrum of AAs (96), both of which may vary considerably, depending on nutritional status. This provides a feedback mechanism in which the γ-glutamyl-AAs produced by GGT enter cerebral capillary endothelial cells and are converted to pyroglutamate, which in turn activates 4 of the 5 Na^+-dependent systems at the abluminal membrane. Because these systems are oriented to remove AAs from the brain in an energy-dependent fashion, its upregulation could provide at least a part of a control mechanism to guard against elevations

of AAs in the brain when their availability is excessive. This is of particular interest with regard to smaller nonessential AAs for which systems A and ASC have a relatively high affinity. Thus, this process may serve to modulate the entry of AAs that serve as neurotransmitters, or their precursors.

Concluding Comments

The present view of the BBB is that cerebral endothelial cells participate actively in regulating the composition of brain extracellular fluid and the AA content of the brain. The luminal and abluminal membranes work in a complementary fashion with the Na^+-dependent transport of AAs occurring at the abluminal membrane, and with facilitative transport at the luminal membrane, or, in the case of LNAAs, at both membranes (97).

Although the BBB determines the availability and therefore the brain content of essential AAs, astrocytes and neurons participate in maintaining the extracellular concentrations. Astrocytes and neurons have Na^+-dependent transport systems capable of transporting NAAs and acidic AAs (98–100). These systems are actively involved in regulating AA concentrations in ECF and are especially important in the maintenance of low concentrations of neurotransmitter AAs 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 composition, and the abluminal membrane is especially important in this role.

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