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Review Article

Synergism between the Two Membranes of the Blood-brain Barrier: Glucose and Amino Acid Transport

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Abstract

Brain capillary endothelial cells, which are connected by extensive tight junctions and are polarized into luminal (blood-facing) and abluminal (brain-facing) plasma membrane domains, form the blood-brain barrier (BBB). The polar distribution of transport proteins mediates glucose and amino acid (AA) homeostasis in the brain. The ability to isolate the luminal and abluminal membranes has permitted the study of each side of the BBB separately in vitro and yielded new information on BBB function. The two membranes have different characteristics. Facilitative transporters were found on both membranes in a position to permit the bidirectional transport of glucose, almost all amino acids and taurine. Na^+-dependent transporters were only found on abluminal membranes. The Na^+-dependent transporters on the abluminal side are capable of removing virtually all amino acids including acidic AA from the extracellular fluid of brain (ECF). The presence of Na^+-dependent carriers on the abluminal membrane provides a mechanism by which the concentrations of AA, glucose and taurine in the ECF of brain may be maintained at optimal levels under physiological and pathophysiological circumstances. Facilitative carriers for glutamine (n) and glutamate (xg^-) are found only in the luminal membrane of the BBB. This organization allows the net removal of acidic and nitrogen-rich AA from brain, and explains the low rate of glutamate and glutamine penetration into the central nervous system. The presence of a g-glutamyl cycle at the luminal membrane and Na^+-dependent AA transporters at the abluminal membrane may serve to modulate movement of AA from blood to brain. The g-glutamyl cycle is expected to generate pyroglutamate within the endothelial cells. Pyroglutamate stimulates Na^+-dependent AA transporters at the abluminal membrane thereby reducing net influx of AA the to brain. It is now clear the BBB may actively participate in the regulation of the AA content of the brain as well as contributing to the control of brain osmolarity.

Keywords: Facilitative transport; Na^+-dependent transport; neutral amino acids; Acidic amino acids; taurine; glucose; membranes; pyroglutamate; osmolytes

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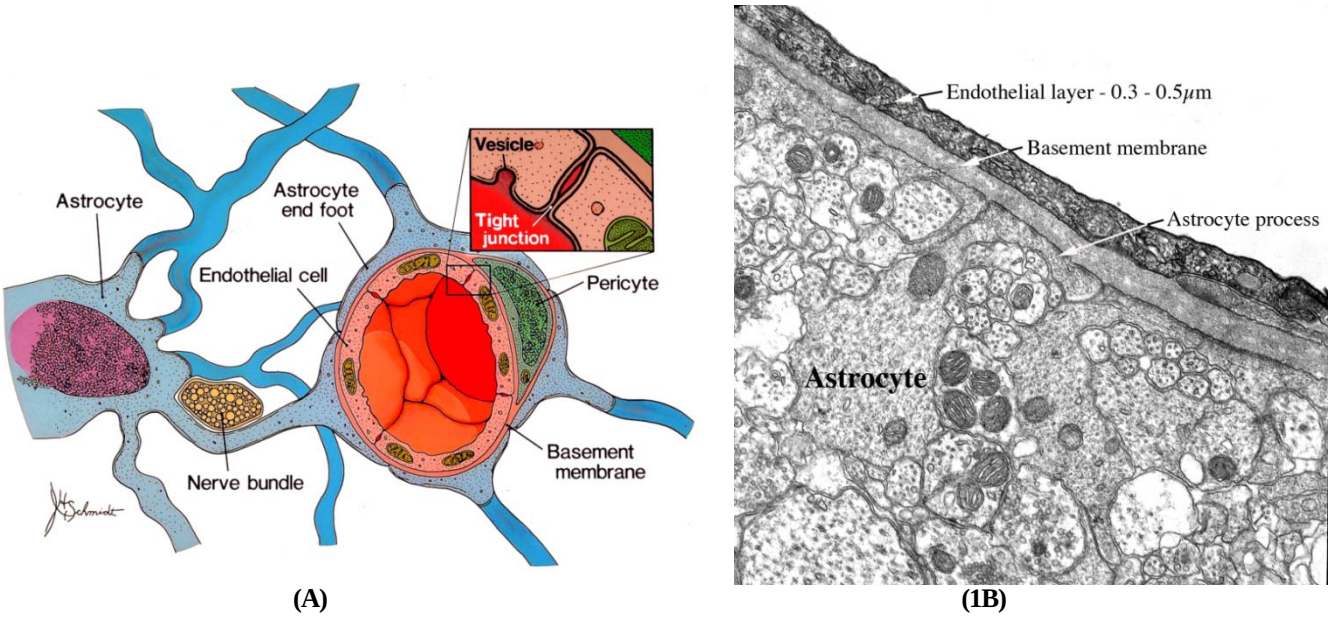
Introduction

The brain is protected from the changing metabolite, ionic and drug concentrations in blood by the blood-brain barrier (BBB) that surrounds the entire central nervous system including the spinal cord. 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 comprising collagen, fibronectin and laminin that completely covers the capillaries, pericytes that are embedded in the basement membrane, and astrocyte processes that surround the basement membrane [1]. Each of these layers could, potentially, restrict the movement of solutes (Figure 1).



(A) **(1B)**

Figure 1 (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 (commonly called end-feet) surround cerebral capillaries (figure was previously published in IUBMB Life). **(B)** An electron micrograph of a cerebral capillary shows the basic elements. The electron micrograph was provided by Robert Page, MD; Professor, Neurosurgery and Anatomy, Pennsylvania State University College of Medicine.

Endothelial cells were demonstrated to be the primary site of the BBB when it was observed that horseradish peroxidase could not pass the endothelial layer from either the blood or the brain [1]. While researchers argued that the astrocytes were a likely site of the barrier [2], the endothelial cells were demonstrated to have high electrical impedance, ≈ 2,000 ohms x cm^2, therefore, even ions are restricted by the endothelial cell layer [3]. It is now accepted that the cerebral endothelial cell layer is the principal site of the BBB in mammals.

Cerebral capillary endothelial cells differ from other mammalian capillary endothelial cells by having fewer cytoplasmic vesicles, more mitochondria and a larger number of tight junctions between overlapping cells [4]. The tight junctions inhibit paracellular movement, prevent membrane molecules from moving from one cell to another, and divide the membranes of the endothelial cells into two distinct sides [5, 6]. Different populations of both lipids and intrinsic proteins (e.g., transporters) exist on the luminal and abluminal sides [7-9]. Thus molecules must pass two sheaths of membrane to enter the brain. The combined characteristics of these membranes determine which molecules traverse the barrier and how fast. Extracellular fluid (ECF) of brain originates from

cerebral capillaries and percolates through the brain parenchyma to join the cerebrospinal fluid (CSF) produced by the choroid plexus [10]. The CSF then flows to the systemic circulation via arachnoid granulations [11]. The BBB has an area of about 12 m^2 -- about 5,000 times that of the choroid plexus [12]. The BBB is the focus of this review because it is the primary pathway by which brain receives nutrition from the circulation.

Various methods are used to study the transport of solutes across the BBB in vivo and in vitro including: single-pass indicator diffusion [13], the brain uptake index [14], in situ brain perfusion [15], isolated brain microvessels [16, 17], and cultured endothelial cells [18-20]. These techniques give valuable information about transport, but they do not distinguish between the different transport properties of the luminal membranes and abluminal membranes.

Transport studies in vivo led to the impression that the BBB, at least with regard to nutrients, was 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 [21]. On the other hand, it was known that ions such as bicarbonate, Na^+, K^+, Cl^-, Ca^{++} etc. are actively secreted across the BBB [2, 11] and are important in regulating ionic content of the ECF [11]. (Na^+/K^+)-ATPase is present in the abluminal membrane capable of maintaining the concentration gradient of Na^+ (external > internal) thereby allowing Na^+-dependent co-transport of nutrients [22].

This review will focus on relatively current knowledge gained by studying the transport properties of the individual membranes of the BBB reviewed earlier [23, 24]. Study of the isolated membranes has allowed the determination of the contributions of the abluminal as well as the luminal membrane to brain nutrient balance.

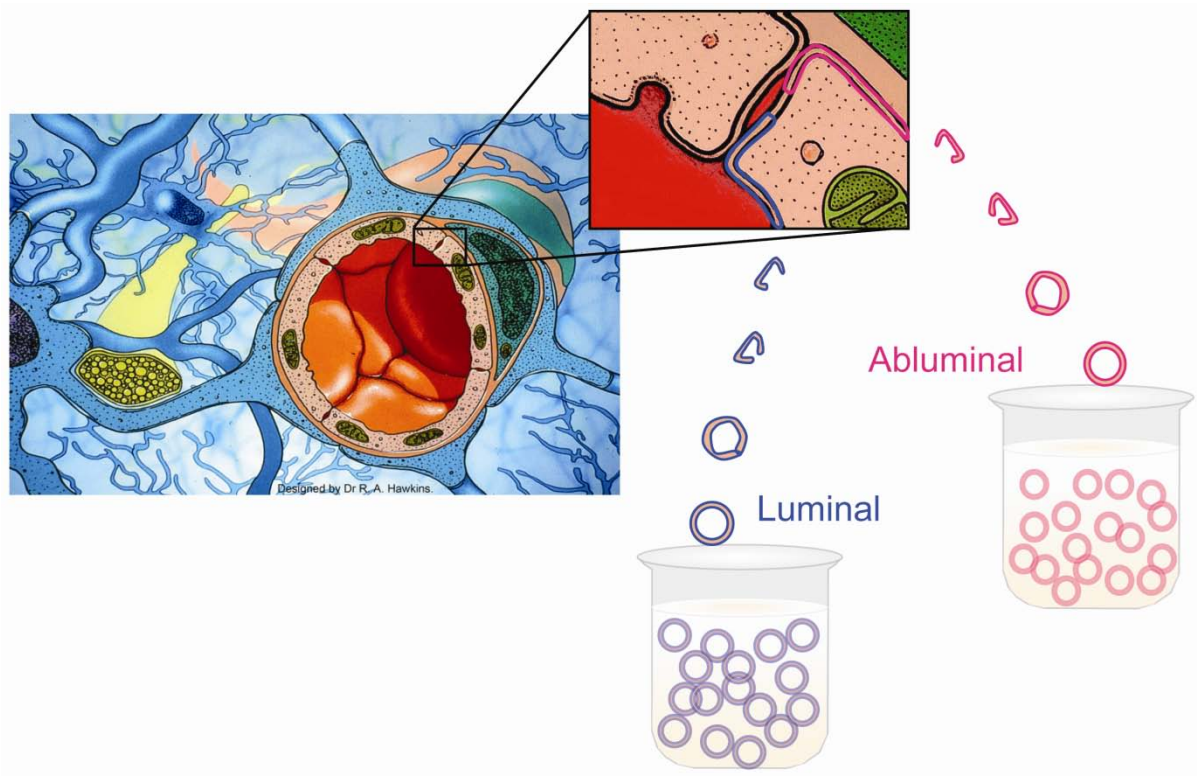


Figure 2 Isolated luminal and abluminal membranes. Capillaries are collected from the bovine cerebral cortex, and their membranes detached [25]. The luminal and abluminal membranes are isolated by differential centrifugation. The membranes form sealed spheres that are suitable for the study of transport [26-28]. It is possible, for instance, to load the vesicles with K^+ thereby creating transmembrane potentials. External Na^+ and substrate can be manipulated as well thereby testing for the presence of Na^+-dependent transport systems.

Isolating Luminal and Abluminal Membranes

Betz and asociates [8] developed a procedure to separate the respective plasma membrane domains: they demonstrated a polarity between the two sides. Sánchez del Pino and colleagues recognized the potential of using these membranes to study transport under controlled conditions [26-28]. 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 (Figure 2). The isolated membranes maintain functional transport properties, and thus may be used to characterize the contribution of each membrane domain to BBB activity under controlled conditions in vitro. For instance, the ionic composition inside and outside the vesicles permits the exploration of such conditions as the influence of the transmembrane potential and Na^+-dependence. This advance allowed the study of the BBB in a different manner and resulted in a change in the concept of the BBB and the synergy between its two membranes. The following sections illustrate that the BBB is an active participant in the regulation of the brain's amino acid (AA), glucose and ammonia contents.

Glucose

Facilitative transport of glucose: Glucose supplies virtually all the fuel for cerebral energy metabolism of the mammalian brain: it is the only substrate able to completely sustain neural activity [29]. Glucose provides energy for neuronal activity through oxidative metabolism, both in the basal and activated states [30]. Cerebral blood flow, oxygen consumption, and glucose delivery are coupled to glucose metabolism [31, 32].

Facilitative diffusion across the BBB is primarily mediated by GLUT1, the first facilitative glucose transporter to be cloned [33]. Two different molecular weight forms of this facilitative transporter (45 and 55 kDa) exist within the brain. The difference in their relative molecular weight is accounted for by a differing extent of glycosylation [34]. However, these species do not appear to differ in their protein structure or kinetic characteristics [34]. The higher molecular weight 55 kDa isoform, which is comparable to that found in erythrocytes of higher mammalian species, is the predominant isoform in the BBB; it is present in both the luminal and abluminal membranes, as well as an intracellular pool [35-40]. The 45kDa GLUT1 isoform is the predominant glucose transporter in glial cells [41].

Certain electron microscopy studies of human and rat BBB have reported an asymmetric distribution of the GLUT1 transporters among compartments with 11% of the transporters residing in the luminal membranes, 44% in the abluminal membranes, and the remaining 45% residing in the intracellular vesicular pool [40, 42] while others found the distribution to be different in canine brain endothelial cells with similar levels of GLUT1 transporters in the luminal and abluminal membranes and a smaller intracellular pool [43]. Our own studies in rat determined GLUT1 to be 22.5% in luminal membranes; 22.5% intracellularly and 55% in abluminal membranes. It should however be noted that the levels of detection of the transporter is very dependent on the fixation conditions and the antibody [39].

Direct kinetic measurements of the transport activity in isolated bovine membranes showed similar activity in both membranes [44], which was consistent with corresponding binding studies with the competitive inhibitor cytochalasin B. However initial western blot analysis suggested a marked discrepancy in the levels of GLUT1 in the respective membranes dependent on the specific antibody. Further examination of isolated membranes using of 2D-PAGE/Western blotting reveal different GLUT1 conformations that arise from differential phosphorylation of GLUT-1 [39]. The role and significance of the phosphorylation of GLUT1 remains to be determined. GLUT3 may be present in

the luminal membranes of canine and bovine endothelial cells (unpublished). Evidence exists that the message for GLUT3 is present in rat endothelial cell transcriptomes [45].

Na-dependent transport of glucose: A Na^+-dependent transporter was discovered on the abluminal membranes of rats with a higher affinity and lower capacity than the facilitative transporter, while Enerson and Drewes identified the transcriptome of a Na^+-dependent glucose transporter (Slc5a2) in rat brain capillaries [44, 45]. The Na^+-dependent glucose transporter is in a position to transport glucose from the ECF into the endothelial cells against a concentration gradient, using the energy of the inwardly directed Na^+ gradient. A model of our concept of the constituents and polarity of glucose transport across the BBB is illustrated in Figure 3.

The discovery of a Na^+-dependent glucose transporter on the abluminal membrane is interesting in view of the fact that the brain requires mechanisms ensuring continuous glucose delivery. On the other hand, it has been observed in all mammalian species studied that the brain glucose concentration is only about 20% that of plasma [41, 46-49]. Although the delivery of glucose to various brain structures is proportional to their metabolic rates, the velocity of unidirectional glucose influx is only about 50-75% greater than the rate of glucose consumption [30]. The question arises whether there is an advantage for the brain to reside in an environment of relatively low glucose concentration? A possible explanation is that the Na^+-dependent transport mechanism prevents glucose from reaching concentrations that could osmotically compromise the extracellular environment or exert cytotoxic effects through, say glycosylation of proteins.

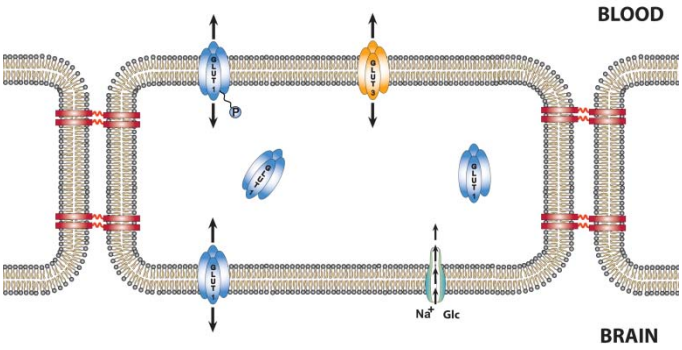


Figure 3 Glucose transporters across the BBB. GLUT1 is located in both the luminal and abluminal membranes with similar activity [39, 44, 50]. GLUT1 on the luminal membrane is, however, phosphorylated and does not react as strongly to antibody to the carboxy terminus. Because of this some authors suggested that GLUT1 was unevenly distributed. GLUT3 may also be in the luminal membranes of endothelial cells (unpublished) and evidence for GLUT3 exists in rat transcriptomes [45, 51].

Amino Acids

Facilitative amino acid transporters existing in both membranes of the BBB: Early studies of AA transport in vivo identified facilitative transporters on the luminal membrane that were saturable and stereoselective [52, 53]. Luminal carriers of AA have no dependence on Na^+ gradients [52, 54-57]. Three broad classes of facilitative carriers exist: large neutral amino acids (LNAA), cationic AA (CAA), and acidic AA (AAA) [58]. Currently, four facilitative carriers have been identified

L1, y^+, xG^-, n and one for taurine. L1 and y^+ are present in both membranes [27, 28] while xG^- and n are restricted to the luminal membrane [59].

Facilitative transport of large essential neutral amino acids; system L1: Early studies of transport in vivo revealed a distinct pattern of LNAA uptake by the brain: movement of essential NAA (neutral amino acids) from blood to brain was greater than non-essential NAA; the movements of the latter were minimal [54, 60]. Transport was facilitative, Na^+-independent and NAA were preferred [58]. Therefore, the carrier seems to belong to the L-system (leucine preferring) originally described by Oxender and Christensen and it is probably the high affinity form currently referred to as L1 [61-64]. Measurements in membranes indicate L1 is present in both membranes in a 2:1 ratio (luminal-to-abluminal) [27, 28]. The substrates carried by L1 include: leucine, valine, methionine, histidine, isoleucine, tyrosine, tryptophan, phenylalanine and threonine, most of which are essential AA. The affinity constants (Km) are in the μM range and similar to the plasma concentrations [64]. Glutamine has also been described as a substrate of L1, but glutamine transport is not completely inhibited by BCH (2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid), a specific inhibitor of the L1 system. Therefore, it seems likely that glutamine is transported by system n as well as L1 [59].

System L1 seems to be the most important path by which essential NAA gain access to the brain. Fernstrom and Wurtman demonstrated the important role of the L1-system and the competition among LNAA by showing that brain tryptophan and serotonin contents were correlated with the ratio of tryptophan-to-LNAA existing in plasma [65]. They concluded that competition between tryptophan and other LNAA for entry to the brain is an important factor in determining the content of serotonin in brain.

Facilitative transport of cationic amino acids: system y^+: Smith concluded that system y^+ is the primary CAA transporter of the BBB from experiments conducted in vivo that examined the BBB only from the luminal side [66]. A more recent study of plasma membranes isolated from bovine brain microvessels allowed characterization of the CAA transporters on both sides of the BBB [67].

Two families of proteins have been identified that transport CAA. CAT or cationic amino acid transporters system (y^+) is selective for cationic amino acids, whereas BAT or broad scope amino acid transport systems ($B^{0,+}$, $b^{o,+}$, and y^+L) also accept neutral amino acids [68-73]. Transporter system $B^{0,+}$ is the only Na^+-dependent carrier that carries CAA, as well as some NAA (although with less affinity). No evidence was found to support the presence of System $B^{0,+}$ in the BBB and no evidence was found of Na^+-dependence of CAA transport [67, 74-76]. In this regard, the CAA are unique because all other naturally occurring AAA and NAA examined to date have Na^+-dependent transporters on the abluminal membrane that are capable of coupling the Na^+ gradient existing between the extracellular fluid of brain (ECF) and BBB endothelial cells to transport AA out of the ECF. Facilitative transport seems to be the only mechanism in the BBB to allow the movement of CAA.

Na^+-independent systems $b^{o,+}$ and y^+L were not found in either membrane [67]. Therefore as posited by Smith, only system y^+ is available to transport CAA [66]. In addition to transporting CAA, y^+ exhibits weak interactions with NAA if Na^+ is present and hence it is referred to as y^+ [77, 78]. In the BBB, y^+ may transport several essential NAA (phenylalanine, threonine, histidine, valine, and methionine) as well as non-essential NAA (serine, glutamine, alanine, and glycine) but the affinity constants are about ten-fold greater than those of system L1 [67]. Thus while y^+ may contribute to the "first-order" transport component observed in studies of AA transport [21], system L1 must be considered the principal provider of essential NAA while y^+ is primarily a purveyor of arginine.

The ability of system y^+ to transport several non-essential amino acids (serine, glutamine, alanine and glycine), with affinity values similar to their plasma concentrations may explain the slight permeability of the BBB to small NAA [21, 60].

While both membranes of the BBB contain y^+, its activity is greater on the abluminal side and it is voltage sensitive [67]. The affinity of y^+ is greater for arginine compared with the other CAA and y^+ is probably important in the provision of arginine for nitric oxide

(NO) synthesis. The biosynthesis of NO requires L-arginine and O_2 for the NO synthase (NOS) catalyzed reaction. Endothelial cells do not have the ability to synthesize arginine de novo [79]. Therefore endothelial cells must rely on an external source of arginine; it is likely that the availability of arginine is the determining factor in NO production by endothelial cells [80]. Three isoforms of NOS have been identified: neuronal (NOS-1), inducible (NOS-2) and endothelial (NOS-3) [80]. Real-time PCR and Western blotting techniques established the presence of all three known NOS in cerebral endothelial cells suggesting that NO can be produced in brain endothelial cells [67].

Facilitative amino acid transporters existing only in the luminal membrane:

Facilitative transport of glutamine; system n: Facilitative transport of glutamine across the luminal membrane of the BBB was not inhibited by BCH and did not demonstrate trans-stimulation [59]. This transport system is similar to system n described in hepatic plasma membrane vesicles [81]. The BBB system n is inhibited by asparagine and histidine [76] as was found in hepatic vesicles by Pacitti et.al. System n exists solely on the luminal membrane.

Table 1 Amino acids transported by facilitative transport systems.

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

AA transported, or shown to inhibit transport, are indicated by the symbol +. Facilitative transport (weak) of NAA by y^+ in the presence of Na^+ are indicated the symbol *. Systems L1 and y^+ exist on both membranes while systems xG^- and n are restricted to the luminal membrane [59]. AA in italics are essential in brain [82]. The membrane distribution of these transporters is shown in Figure 4.

Table 2 Kinetic characteristics of facilitative amino acid transporters on the blood-brain barrier.

Transporter (Substrate)	Apparent Km (μM)	Apparent Vmax ($pmol*mg^{-1}*min^{-1}$)	Clearance ($\mu l*mg^{-1}*min^{-1}$)	Position
L1 (Phe)	12	94	8	Luminal & Abluminal
y^+ (Lys)	800	5,800	7	Luminal & Abluminal
n (Gln)	1000	1,100	1	Luminal
xG^- (Glu)	900	700	1	Luminal
Tau	0.06	0.1	2	Luminal & Abluminal

The radiolabeled substrates used for measurements are in parenthesis. Clearance was calculated to the nearest integer as Vmax/Km. Values were taken from [26-28] except taurine, which was from [83].

Table 3 Amino acids transported by Na^+-dependent systems of the abluminal membrane.

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

AA that are transported, or shown to inhibit transport, are indicated by a +. Data for systems A, N, and ASC are from [84]. Data for Na^+-LNAA are from [75]. Data for the EAAT1-3 family are from [85]. AA in italics are essential in brain [82]. The Na^+-dependent transporter for taurine has not yet been characterized in the BBB.

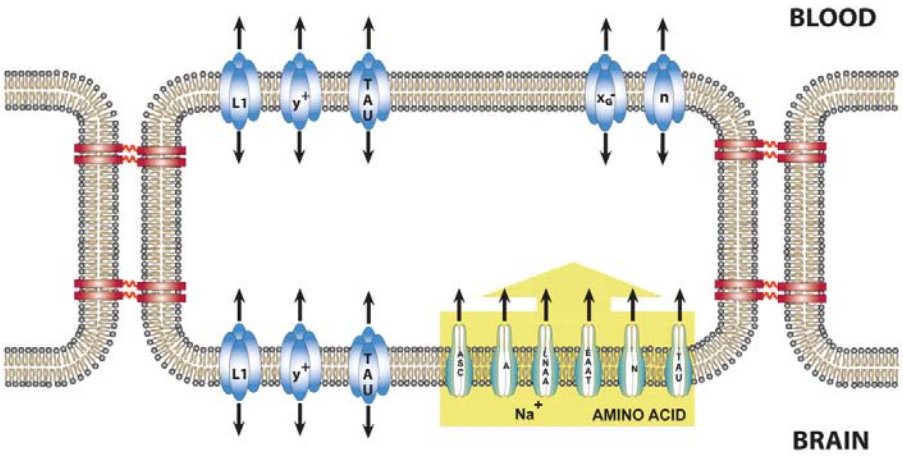


Figure 4 Amino acid transporters of the BBB. The brain gains access to all essential AA through the facilitative systems L1 (NAA), y^+ (CAA) and TAU that exist on each membrane. Facilitative transporters xG^- and n exist only on the luminal membrane and are in a position to allow glutamate, aspartate and glutamine egress. Each facilitative transporter carries several substrates (please see Table 1). The Na^+-dependent transport systems provide mechanisms for the removal of non-essential AA, toxic AA, as well as maintaining the optimal concentrations of all other AA. As with the facilitative systems there is considerable overlap of substrates (please see Table 3). All naturally occurring NAA are transported by at least one system and some by as many as three. Abbreviations: A, Na^+-dependent system A; N, Na^+-dependent system N; ASC, Na^+-dependent system ASC; Na^+LNAA, Na^+-dependent system Na^+LNAA, EAAT, Na^+-dependent glutamate transporter, TAU, Na^+-dependent taurine transporter, xG-, facilitative glutamate transporter, n, facilitative glutamine transporter.

Facilitative transport of acidic amino acids; system xG^-: Benrabh and Lefauconnier [86] studied glutamate uptake in vivo and found no evidence of Na^+-dependent transport when glutamate was presented to the luminal membrane. They concluded the carrier was facilitative and probably the xG^- form because no evidence for the cystine-glutamate exchanger xC^- could be found. This was confirmed in isolated luminal membranes. Cystine did not compete with glutamate for uptake while aspartate did. Furthermore, cystine did not accelerate glutamate uptake in vesicles preloaded with cystine [59]. Facilitative glutamate transport was only found on the luminal border allowing the release of glutamate from endothelial cells to the plasma [59]. 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 4.

Facilitative transport of Taurine: Taurine, (2-amino-ethanesulfonic acid) is one of the most abundant amino acids in mammals [87]. (Taurine is commonly considered an amino acid although amino acids are molecules containing both an amino and a carboxyl group.) Taurine is synthesized primarily by the liver, [88] and enters and leaves the brain via transporters [89-91]. Brain has a high taurine content comparable to amino acids such as glutamine, aspartate and gamma amino butyric acid (GABA) [92]. Taurine has multiple cellular functions including a central role as a neurotransmitter and as an important osmolyte [93]. Taurine is almost completely negatively charged at pH 7.4. Rasgado-Flores et. al. studied taurine transport across both membranes of the BBB [83]. They confirmed the presence of facilitative transporters in both BBB luminal and abluminal membranes with affinity constants in the µM range however the transport system has yet to be identified.

Amino acid gradients between brain and plasma: The concentrations of all naturally occurring AA in CSF (presumably similar to the ECF) are about 10% or less than the plasma concentrations; the exception is glutamine that has a similar concentration in CSF and plasma [82]. This situation cannot be explained by the consumption of AA by the brain because the arteriovenous differences across the brain of most AA are almost imperceptible [94, 95] as are the

arteriovenous differences of ammonia (NH_4^+), a by-product of AA catabolism [96]. These observations indicate that AA 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" [97].

Na^+-dependent transport systems of the abluminal membrane: Several Na^+-dependent systems have been identified in the abluminal membrane of the BBB. They include: A (alanine preferring), which was first characterized and shown to actively transport small non-essential NAA [7, 26, 84], ASC (alanine, serine and cysteine preferring) [84, 98-100], N (glutamine, asparagine and histidine preferring) [59, 84], the excitatory acidic AA family (EAAT, aspartate and glutamate preferring) [85, 101], a system that transports primarily essential AA, LNAA [75] and a transporter of taurine [83].

Na^+-dependent transport of AA exists only in abluminal membranes. No Na^+-dependency has been detected in luminal membranes, which appear to have only facilitative carriers. Therefore, the Na^+-dependent transporters are in a position to remove AA from 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., [26, 28] found Na^+-dependent phenylalanine transport that was inhibited by BCH. Studies by Van Winkle and associates [68] had demonstrated system $B^{0,+}$ as a Na^+-dependent carrier that recognizes NAA and is inhibited by BCH. Because of this characteristic and the observed inhibition, the authors thought carrier system $B^{0,+}$ was likely to be responsible for the transport activity. A characteristic of system $B^{0,+}$ is the ability to transport CAA [68]. However, the rate of lysine transport was not inhibited by the presence of BCH casting doubt on the presence of system $B^{0,+}$ [75]. Further investigation led to the discovery of Na^+-LNAA as the carrier responsible for the BCH-inhibited, Na^+-dependent phenylalanine transport and other LNAA [75].

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 [75]. Na^+-LNAA has a high-affinity for leucine and is inhibited by other NAA including: glutamine, histidine, methionine, phenylalanine, serine, threonine, tryptophan, and tyrosine. Transport is Na^+-dependent, voltage sensitive and inhibited by BCH. The spectrum of AA carried by Na^+-LNAA is similar to the facilitative system L1 that allows the entry of essential LNAA down their concentration gradients (compare Tables 1 and 3). The presence of a Na^+-dependent carrier on the abluminal membrane, capable of removing LNAA from the brain, most of which are essential may provide a mechanism for the control of the LNAA content of the brain.

Na^+-dependent transport of small non-essential neutral amino acids; system A: The activity of system A, named for its preference for transporting alanine may be distinguished from other Na^+-dependent carriers by its acceptance of MeAIB (N-methylamino-isobutyric acid) as a unique substrate [61, 102]. System A is voltage sensitive; three positive charges are translocated per MeAIB molecule [84]. System A is inhibited by small non-essential AA such as: proline, alanine, histidine, serine, asparagine and glutamine as well as the essential AA histidine. Some laboratories reported a similar AA spectrum for system A but also included glycine [7, 61]. Glycine transport was not mediated by system A in isolated membrane vesicles but was a putative substrate of system ASC.

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 [98-100]. In addition to alanine, serine, cysteine and glycine, several essential AA were putative substrates including: methionine, valine, leucine, isoleucine, and threonine. ASC activity is independent of the transmembrane potential [84].

Na^+-dependent transport of nitrogen rich amino acids; System N: System N has a preference for NAA that are nitrogen-rich such as glutamine, histidine, and asparagine, hence its designation [103, 104]. BBB abluminal membranes also transport serine via this system. System N was not affected by the transmembrane potential [84]. Li^+ could substitute for Na^+ suggesting system N in the BBB is similar in to the system N in liver cells.

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 Km of 14 µM at a transmembrane potential of –61mV [85]. Analysis of mRNA demonstrated three 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. The activity of the three transporters was 1:3:6, EAAT1: EAAT2: EAAT3, respectively. EAAT4 may also be present as the transcriptome has been found in rat brain endothelial cells [45]. Collectively the EAAT family is the most powerful of the Na^+-dependent AA transporters; they show the greatest ability to clear AA at low concentrations (Table 4).

Na-dependent transport of taurine: Na^+-dependent taurine transport was found only on the abluminal membrane. This indicates that taurine can be extruded from the brain even in the absence of a favorable concentration gradient for taurine across the abluminal membrane. The Na^+-dependent transport of taurine was voltage dependent between -25 and -101 mV and had an affinity constant in the low µM range (Table 4). Na^+-dependent transport of taurine was sensitive to external osmolality being most active at low osmolalities [83].

Table 4 Kinetic characteristics of Na^+-dependent amino acid transporters in abluminal membranes.

Transporter (Substrate)	Apparent Km (µM)	Apparent Vmax (pmol*mg^{-1}*min^{-1})	Clearance (µl*mg^{-1}*min^{-1})	Voltage Sensitivity
A (MeAIB)	400	500	1*	Yes
N (Gln)	1,300		4,400	No
ASC (Ala)	110	660	6	No
Na^+-LNAA (Leu)	21	114	5*	Yes
EAAT (Glu)	14	151	11*	Yes
Taurine	7	7	10	Yes

The radiolabeled AA used for measurements are in parenthesis. Clearance was calculated as Vmax/Km. Kinetic values were from: Na^+-LNAA, [75]; EAAT1-3, [85] A, ASC and N [84] and taurine [83]. Values marked by an asterisk were measured at a calculated transmembrane potential of -61 mV (taurine -59mV). MeAIB (20 mmol x l^{-1}) was included in measurements of systems N and ASC to exclude transport by system A.

Possible Physiological Relevances

Organization of the various transport systems: The brain gains access to all essential AA through the facilitative systems L1 and y^+. There is considerable substrate overlap within the facilitative systems as well as within the Na^+-dependent systems (Tables 1 and 3). Facilitative transport of taurine also exists, but the carrier has not been characterized in the BBB.

Six Na^+-dependent AA transport systems are present exclusively on the abluminal membrane of the BBB (Figure 5) and the capacities of these transporters are similar 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 AA from the brain extracellular fluid to the blood [105, 106].

Thus, AA 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 AA in the brain.

The Na^+-dependent transport systems provide a mechanism for the elimination of non-essential AA and toxic AA as well as maintaining the optimal concentrations of all other AA. All naturally occurring AA are transported by at least one system and some by as many as three (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 branched chain AA (BCAA) may influence brain function and affect: appetite [107], physical and mental fatigue [108-110], mental performance [111], physical endurance [112, 113], sleep [111] hormonal function, blood pressure, and affective state [114]. Presumably, BCAA influence brain function by altering the availability of aromatic AA [65]. As mentioned, transport of LNAA is mediated by the facilitative system L1, which is shared by several LNAA with BCAA being more effective in competition with aromatic AA. Consequently, when plasma BCAA concentrations rise, which can occur in various normal and abnormal situations, they impair the entry of aromatic AA, notably tryptophan [114]. Serotonin synthesis in brain depends directly on the availability of tryptophan. Therefore, when plasma BCAA concentrations rise, the contents of brain tryptophan and serotonin fall [114]. While 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 adds a new element that should be considered [75, 84]. These Na^+-dependent carriers are capable of propelling all NAA, including BCAA and aromatic AA back toward the plasma against their ECF-plasma concentration gradient and may affect the brain content of AA.

Glutamate in plasma and brain: Glutamate, a non-essential amino acid, is the most abundant free AA in the brain. In CNS glutamate functions as a neurotransmitter, a link between the redox states of the pyridine nucleotides (NAD^+ and $NADP^+$), and as a fuel reserve. The oxidation of glutamate to oxaloacetate yields 12 ATP per molecule. Therefore, when the brain has insufficient glucose levels or glycolytic flux is reduced, it mobilizes glutamate as a fuel [115-117].

Compartmentation of glutamate: In the CNS glutamate exists as the free AA divided between two separate metabolic compartments located in astrocytes and neurons. These compartments were first recognized in the brain on the basis of radioisotope precursor-product relationships between glutamine and glutamate [96, 118-121]. Compartmentation is almost absent at birth and develops in parallel with glial cells since glutamine synthetase is found only in astrocytes.

Neuronal glutamate is contained in at least two pools, in neuronal perikarya and dendrites and the other in nerve terminals (vesicles) [117, 120, 121]. Nerve impulses trigger release of glutamate from the pre-synaptic terminal, which in turn binds to the glutamate receptors on the opposing synaptic membrane. Neurotransmission is terminated by astrocytes uptake and neurons [122].

Excitotoxicity hypothesis of neuronal death: Early studies that used pharmacological doses of glutamate demonstrated brain damage in areas of the brain that were not protected by the BBB [123, 124]. These studies led to the concept that neuronal death could be produced by over stimulation of excitatory AA receptors [125-127]. Subsequently, this hypothesis became a popular explanation of the pathogenesis of neuronal death in a variety of acute conditions. However, the source of glutamate arises from within the brain. For instance, during an ischemic episode, release of glutamate from brain cells may result in an excessive concentration of glutamate in the ECF [128-131]. The excitation of neurons by glutamate may in turn result in the opening of receptor-coupled ionophores, of which calcium channels are of particular importance. A large influx of calcium associated with impaired intracellular calcium sequestration mechanisms, that activate catabolic enzymes, may ultimately result in neuronal

death [132].

Glutamate in circulation: Plasma glutamate concentrations are in the range of 50 to 100 μM in humans and other species [82]. Even when relatively large quantities of monosodium glutamate are added to food of mice, monkeys or humans only small changes in the plasma concentration of glutamate were found [133-136].

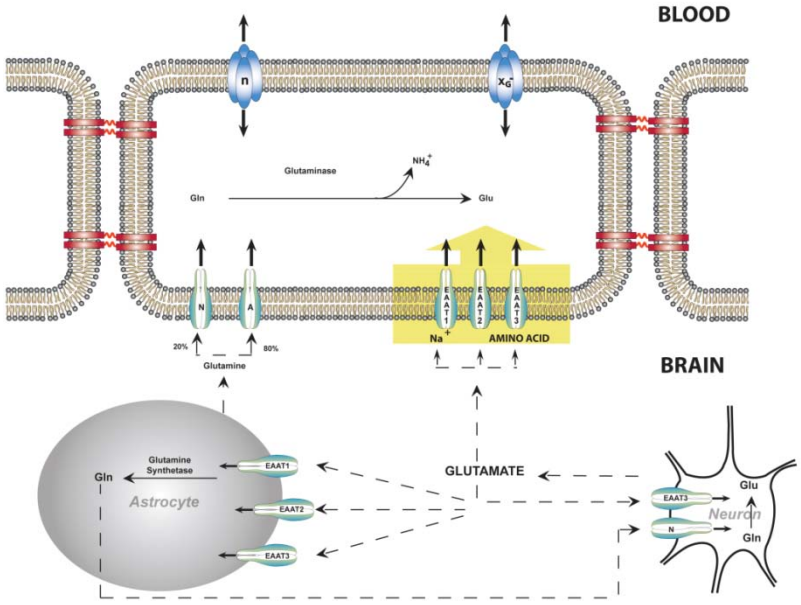


Figure 5 Glutamate and glutamine transport between neurons, astrocytes, and endothelial cells. The presence of Na^+-dependent carriers capable of pumping glutamine and glutamate from brain into endothelial cells, glutaminase within endothelial cells to hydrolyze glutamine to glutamate and $NH4^+$, and facilitative carriers for glutamine and glutamate at the luminal membrane provides a mechanism for removing nitrogen and nitrogen-rich AA from the brain [59]. EAAT1, 2 and 3 are present in endothelial cells, and astrocytes [85, 137, 138]. A transcript of EAAT4 has also been found in endothelial cells indicating that it may be present as well [45]. EAAT3 is present in nerve cells [139]. Abbreviations: A, Na^+-dependent system A; N, Na^+-dependent system N; EAAT, Na^+-dependent glutamate transporter, xG^-, facilitative glutamate transporter, n, facilitative glutamine transporter.

Control of extracellular glutamate in the BBB: Early studies of the BBB using whole brain perfusions or animals in vivo identified facilitative transporters in the BBB membrane that are saturable and stereoselective [14, 60]. Because the substrate was presented to the capillary lumen it may be deduced that these transporters are present at least in the luminal membrane. On the other hand, it has been shown in several studies that glutamate does not enter the brain in significant quantities, except in the circumventricular organs [140-142]. Until recently this was puzzling. Why should there be a transport system for an AA that is synthesized within the brain in large quantities? Examining the luminal and abluminal membranes separately provided an explanation.

As describe above, facilitative glutamate transport exists solely in the luminal border in a position to allow transport in both directions between plasma and endothelial cells [59]. However, the function of a transporter for AA that had a high affinity and a low capacity was not clear: both glutamate and aspartate are non-essential amino acids that are synthesized and accumulated in high concentrations in the brain [21, 143, 144].

Active transport systems expel glutamate from the ECF: Ordinarily, ECF glutamate is kept very low≈(0.5-2 μM) [117]. In fact the concentration of glutamate and aspartate in cerebrospinal fluid is lower than any other AA [82]. The large gradient between brain cells and ECF is maintained by EAAT, which couple the steep Na^+ gradient that normally exists between the ECF and brain cells. Currently five members of the

EAAT family have been identified [117, 145, 146]. They reside in the plasma membranes of astrocytes, neurons and the BBB [85, 117, 131, 139, 147-152]. The Na^+-dependent transporters work at the limit of their ability to maintain the glutamate gradient between the brain cells and ECF. If the oxygen supply is insufficient to maintain ATP levels, membrane Na^+/K^+-ATPase cease to function. Under these circumstances the Na^+ gradient is dissipated and glutamate is released from both astrocytes and neurons by reversal of the EAAT family of transporters. If ECF glutamate rises nerve cells may be damaged.

Current concept of glutamate transport across the BBB: The current concept is that when glutamate concentrations increase above optimal in the ECF, the abluminal membrane of the BBB brings glutamate into the endothelial cells. The facilitative transport system in the luminal membrane allows glutamate egress to the circulation (Figure 5).

The organization of the BBB explains why various investigators have found glutamate entry to brain is almost undetectable [94, 140-142, 144]. Glutamate may enter the endothelial cells, but net movement of glutamate from endothelial cells to the brain is nearly impossible. This is a consequence of the steep Na^+-gradient that powers the EAAT family of glutamate transporters at the border between the ECF and the abluminal membrane of the endothelial cells. Because of this organization the BBB is virtually impermeable to the net movement of glutamate from circulation into the brain.

Ammonia Balance

The organization of the BBB also provides an explanation for a long-standing question regarding brain NH_4^+ metabolism. Various measurements have shown that 20-50% of the NH_4^+ circulating through the blood vessels in brain passes the BBB and is incorporated quantitatively into the amide group of glutamine by astrocytes [96, 119]. It is intriguing, however, that it has not been possible to consistently measure arteriovenous differences of NH_4^+ [96]. If there were no mechanism for the removal of glutamine it would accumulate in brain, thereby raising the osmolarity and causing brain swelling. For instance, taking cerebral blood flow to be 1 ml x min^{-1} x g^{-1} and plasma $NH4^+$ to be 50-100 µmol/ml it may be calculated that glutamine accumulation could be 14-72 µmol/g each day. Clearly this would be an osmotic challenge for the brain. The situation is now clearer. Glutamine may be pumped from ECF into endothelial cells and is at least partially metabolized to $NH4^+$ and glutamate. The remaining glutamine as well as NH_4^+ and glutamate are free to diffuse across the luminal membrane into the blood. This provides an explanation why the rate of NH_4^+ uptake and release are balanced.

This new knowledge also explains how the entry of glutamine (and glutamate) to the CNS is restricted [141, 153, 154] even though carrier activities for both amino acids have been described [58, 144, 155]. Glutamine and glutamate can traverse the luminal membrane on facilitative systems. However, movement into the brain, across the abluminal membrane, is small because of the lack of facilitative carriers in the abluminal membrane. Furthermore, the Na^+-dependent carriers in the abluminal membrane that are driven by the steep Na^+ gradient that exists between brain ECF and the cell interior 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 the brain but also actively export these amino acids and $NH4^+$ 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 $NH4^+$.

Oxoproline Stimulates Na^+-dependent Carriers

The γ-glutamyl cycle produces oxoproline. The γ-glutamyl cycle proposed by Meister, which is important for the synthesis and degradation of reduced glutathione (GSH), has been shown to influence AA transport in various tissue [156, 157]. 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 placenta of pregnant rats showed that oxoproline (also known as pyroglutamate), an intermediate of the γ-glutamyl cycle, serves to stimulate Na^+-dependent AA transport [158, 159].

The first reaction of the cycle occurs extracellularly and is catalyzed by γ-glutamyl transpeptidase (GGT) (Figure 6) [157]. The substrates for GGT are glutathione, which is exported across the luminal membrane of endothelial cells to the plasma side, and extracellular AA in the plasma. The γ-glutamyl-AA that results is transported into the cells. Intracellularly, γ-glutamyl-AA are substrates of γ-glutamyl cyclotransferase, which converts the γ-glutamyl-AA into oxoproline and the corresponding free AA. Subsequently, oxoproline is hydrolyzed to glutamate by oxoprolinase [160].

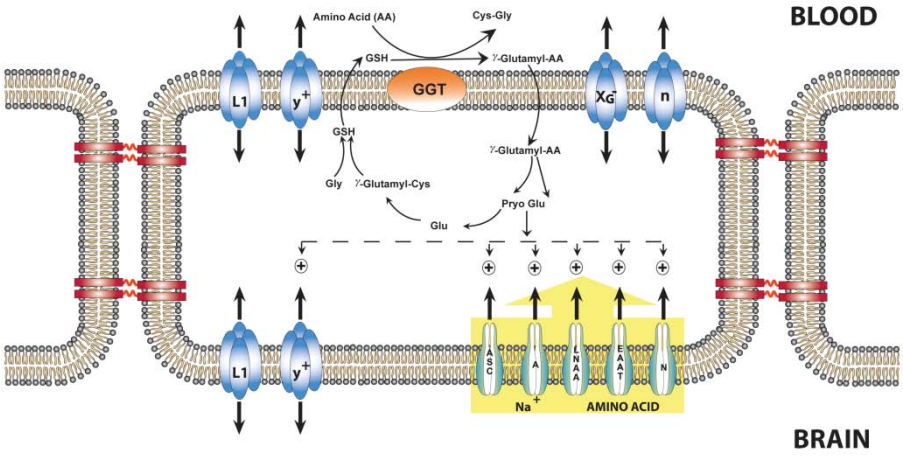


Figure 6 The influence of oxoproline on AA transport across the blood-brain barrier. γ-Glutamyl-AAs are formed at the outer surface of luminal membranes of the endothelial cells by GGT that transfers the γ-glutamyl moiety of glutathione to most AA thereby forming a γ-glutamyl-AA. The γ-glutamyl-AA enters endothelial cells where the AA is released and oxoproline is formed. The Na^+-dependent transport systems A, ASC, and Na^+-LNAA, EAAT and y^+, all located on the abluminal side, are activated by oxoproline. N was the only system not stimulated. L1 is present on both the luminal and abluminal membrane and is not affected by oxoproline [161]. Abbreviations: A, Na^+-dependent system A; N, Na^+-dependent system N; EAAT, Na^+-dependent glutamate transporter, xG^-, facilitative glutamate transporter, n, facilitative glutamine transporter. The possibility exist that oxoproline causes an increase in the transmembrane potential therefore providing a greater driving force.

Oxoproline stimulates the Na^+-dependent system A, but has no effect on facilitative transport of L-phenylalanine (a representative substrate of the facilitative transport system L1); the effect of oxoproline is restricted to the Na^+-dependent AA transport systems of the abluminal membrane [59]. Further studies showed that oxoproline stimulated all Na^+-dependent AA transport systems with the exception of system N, which transports glutamine [84]. The latter is interesting because glutamine is the only AA present in similar concentrations in plasma and ECF and is synthesized from $NH4^+$ that enters brain continuously [96]. Also of interest was the finding that oxoproline stimulated y^+, which not only transports CAA, but also transports a range of NAA in the presence of Na^+ [77, 78].

The presence of GGT in the BBB has been puzzling. GGT activity is high in tissues that actively transport AA, such as the brush border of the proximal convoluted tubules of the kidney, the lactating mammary gland, and the apical portion of the intestinal epithelium [162]. The BBB differs from these tissues in that it is not associated with active AA uptake from plasma. While brain requires essential AA for its

function and growth, their supply is not much greater than the demand, and it is difficult to detect arteriovenous differences of AA across the brain [94, 95, 140]. The data support the hypothesis that the γ-glutamyl cycle influences AA transport systems indirectly through oxoproline, produced intracellularly as an intermediary metabolite of the γ-glutamyl cycle. Oxoproline, in turn acts to stimulate Na^+-dependent AA transport systems. The γ-glutamyl cycle and GGT may serve to monitor the availability of AA to the brain, and constitute the first step in a control mechanism that influences the accessibility and content of brain AA (Figure 6).

The transpeptidation activity of GGT is a function of the plasma concentration and spectrum of AA [163], both of which may vary considerably, depending on nutritional status. This provides a feed-back mechanism in which the γ-glutamyl-AA produced by GGT enter cerebral capillary endothelial cells and are converted to oxoproline, which in turn activates four of the five Na^+-dependent system at the abluminal membrane. Since these systems are oriented to remove AA from the brain in an energy-dependent fashion, up-regulation could provide at least a part of a control mechanism to guard against elevations of AA in brain when their concentration is excessive. This is of particular interest with regard to smaller non-essential AA for which systems A and ASC have a relatively high affinity. Thus, this process may serve to modulate the entry of AA serving as neurotransmitters, or their precursors.

Volume Regulation

Influence of the blood-brain barrier on brain volume: The BBB is an important structure through which the brain may respond to osmotic stresses. The brain is separated from plasma by a continuous layer of endothelial cells that behave in a matter analogous to a single semipermeable membrane. Therefore the brain as a whole acts as an osmometer, shrinking and swelling in response to changes in plasma tonicity [164] and/or in the intracellular osmolyte concentration [165]. For example, brain shrinkage may result from exposure to a hypertonic extracellular environment. On the other hand, brain swelling may occur either from a reduction of plasma osmolality or an increase in the intracellular osmolyte concentration of brain cells. An example of the first instance is hyponatremia [166] where the extracellular Na^+ concentration drops to 135 mEq/L (135 mM) or less [167, 168]. An example of brain swelling resulting from an increase in the intracellular osmolytes concentration is hyperammonemia. In this instance ammonia permeates the cell membranes and gets metabolized in the intracellular milieu of nerve cells yielding the impermeant glutamine [165, 169]. Osmotically obliged water influx leading to cell swelling follows this increase in the intracellular osmolyte concentration. Brain swelling can be rapidly reversed by the injection of impermeant osmolytes such as urea, mannitol or glycerol into the circulation demonstrating that the BBB allows the ready passage of water. Aquaporins 1 and 4 have been identified in choroid plexus and astrocytes respectively and AQP9 in glia and neurons, but aquaporins have not yet been found in the BBB despite the physiological evidence of rapid water movement [170, 171].

The main organic intracellular osmolytes in rat and human brains are glutamate, glutamine, taurine and glycine (Table 5) [166]). Exposure to hypo-osmotic conditions leads to osmotically obliged movement of water into the brain cells producing cell swelling. This event leads to activation of cell volume sensors (e.g., macromolecular crowding, [172]) followed by the promotion of the loss of intracellular osmolytes and osmotically obliged water. This mechanism is called regulatory volume decrease. The first osmolytes to be lost are inorganic, i.e., Na^+, K^+ and Cl^-. This effect however, compromises the excitability of nerve cells. Thus, under the persistent conditions of extracellular hypo-osmolarity (e.g., hyponatremia, reviewed by [166]), there is recovery of the intracellular content of Na^+ and K^+ accompanied by the simultaneous loss of organic intracellular osmolytes. These main osmolytes are: glutamate, glutamine, taurine, myo-inositol and creatine. Interestingly, under persistent conditions of extracellular hypo-osmolarity, the intracellular content of most organic osmolytes recovers partially except for taurine, which keeps exiting the brain cells until being completely depleted. Taurine transport across the

membrane of nerve cells and the blood brain barrier has received considerable attention [83, 173-178] due to the fact that it is one of the most inert osmolytes, has the lowest osmolarity threshold for its exit from the brain cells, and has the largest efflux of all osmolytes. Interestingly, each of the organic osmolytes has facilitative transport systems in the luminal membrane that allow entry to and exit from the brain, as well as Na^+-dependent co-transporter systems on the abluminal membranes in a position to move metabolites from ECF to endothelial cells. Of special interest is the fact that the abluminal transport of taurine across the blood brain barrier is sensitive to osmolarity [83]. The transport rate of this osmolyte is highest under hypo-osmotic conditions (i.e., 229 mOsm/kg H_2O). From a teleological perspective this observation may suggest that this mechanism prevents excessive accumulation of taurine in the interstitial fluid once this osmolyte has been released to prevent brain cell swelling under hypo-osmotic conditions

Table 5 Major osmolytes within rat brain.

Osmolyte	Concentration ($nmol*g^{-1}$)	Na-dependent export on abluminal membrane	Facilitative transport on luminal membrane
Glutamate	12,000	yes	yes
Glutamine	5,590	yes	yes
Taurine	5,500	yes	yes
Aspartate	2,700	yes	yes
Ser, Gly, Ala	2,270	yes	yes
GABA	1,990	yes	yes
Glucose	2,500	yes	yes

All values for metabolites were from control rats studied by [92]. For transport details about each amino acid please see above. GABA transport has not been studied at the level of isolated membranes, however, physiological experiment describe the presence of active efflux [179].

Conclusions

The current view of the BBB is that cerebral endothelial cells participate actively in regulating the composition of brain extracellular fluid as well as the glucose, AA and the ammonia content of the brain. The luminal and abluminal membranes work in a synergistic fashion with Na^+-dependent transport of AA and glucose occurring at the abluminal membrane and facilitative transport at the luminal.

While the BBB determines the availability and therefore the brain content of essential AA, astrocytes and neurons participate in maintaining the extracellular concentrations. Astrocytes and neurons have Na^+-dependent transport systems capable of transporting NAA and acidic AA. These systems are actively involved in regulating AA concentrations in the ECF and are especially important in the maintenance of low concentrations of neurotransmitter AA 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 context.

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