

The blood-brain barrier and glutamate¹⁻⁴

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ABSTRACT

Glutamate concentrations in plasma are 50–100 $\mu\text{mol/L}$; in whole brain, they are 10,000–12,000 $\mu\text{mol/L}$ but only 0.5–2 $\mu\text{mol/L}$ in extracellular fluids (ECFs). The low ECF concentrations, which are essential for optimal brain function, are maintained by neurons, astrocytes, and the blood-brain barrier (BBB). Cerebral capillary endothelial cells form the BBB that surrounds the entire central nervous system. Tight junctions connect endothelial cells and separate the BBB into luminal and abluminal domains. Molecules entering or leaving the brain thus must pass 2 membranes, and each membrane has distinct properties. Facilitative carriers exist only in luminal membranes, and Na^+ -dependent glutamate cotransporters (excitatory amino acid transporters; EAATs) exist exclusively in abluminal membranes. The EAATs are secondary transporters that couple the Na^+ gradient between the ECF and the endothelial cell to move glutamate against the existing electrochemical gradient. Thus, the EAATs in the abluminal membrane shift glutamate from the ECF to the endothelial cell where glutamate is free to diffuse into blood on facilitative carriers. This organization does not allow net glutamate entry to the brain; rather, it promotes the removal of glutamate and the maintenance of low glutamate concentrations in the ECF. This explains studies that show that the BBB is impermeable to glutamate, even at high concentrations, except in a few small areas that have fenestrated capillaries (circumventricular organs). Recently, the question of whether the BBB becomes permeable in diabetes has arisen. This issue was tested in rats with diet-induced obesity and insulin resistance or with streptozotocin-induced diabetes. Neither condition produced any detectable effect on BBB glutamate transport. *Am J Clin Nutr* 2009;90(suppl):867S–74S.

INTRODUCTION

The brain is insulated from the plasma by the blood-brain barrier (BBB), which surrounds the entire central nervous system including the spinal cord (**Figure 1**). The BBB is necessary to provide an optimal chemical environment for cerebral function. Several layers exist between blood and the brain: capillary endothelial cells, a basement membrane that completely covers the capillaries, and finally, astrocyte processes that enclose the basement membrane. Each of these layers could potentially restrict the movement of solutes (**Figure 2**).

Endothelial cells were shown to be the site of the BBB in mammals (2–4) (**Figure 3**). The BBB has high electrical impedance ($\approx 2000 \Omega \cdot \text{cm}^2$); therefore, even the passage of ions is restricted by the endothelial cell layer (5). Cerebral capillary endothelial cells differ from other mammalian capillary endothelial cells by having few cytoplasmic vesicles, more mitochondria (6), and a denser network of tight junctions between

overlapping cells. The tight junctions restrict the movement of molecules between cells and also prevent membrane molecules from moving from one endothelial cell to another (7). Furthermore, the tight junctions divide the membranes of the endothelial cells into 2 discrete sides (8). Different populations of both lipids and intrinsic proteins (eg, transporters) exist in the luminal and abluminal membranes (9–11). Thus molecules must pass 2 sheaths of membrane to enter the brain. It is the combined characteristics of these 2 membrane domains that determine which particles traverse the barrier and how quickly.

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 (12). However, it was known that active transport of ions exists. Bicarbonate and other ions are actively secreted across the BBB (13, 14). Na^+/K^+ -ATPase is present in the abluminal membrane, presumably to mediate blood-to-brain sodium flux. Mg^{++} and Ca^{++} ATPases exist in the BBB as well (15). Also, as mentioned, the BBB has a high capacity for energy production (6).

GLUTAMATE IN THE BRAIN AND THE CIRCULATION

Glutamate, a nonessential amino acid, is the most abundant free amino acid in the brain. In the central nervous system, glutamate functions as a neurotransmitter, as a link between the redox states of the pyridine nucleotides (NAD^+ and NADP^+), and as a fuel reserve. Glutamate metabolism is closely linked to the Krebs cycle. The reaction $\text{glutamate} + \text{NAD}^+ \leftrightarrow \alpha\text{-ketoglutarate} + \text{NADH} + \text{H}^+$ is catalyzed by glutamate dehydrogenase. This mitochondrial reaction, which uses either NAD^+ or NADP^+ as cofactors, is believed to be at equilibrium and therefore serves to assure that the redox state of both pyridine nucleotides is similar

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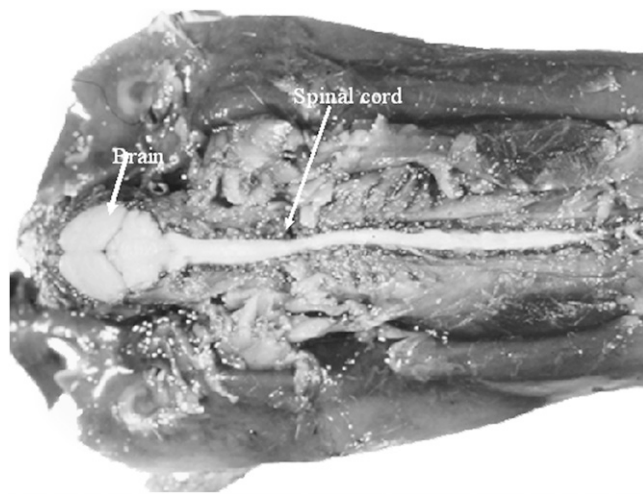


FIGURE 1. The blood-brain barrier exists around the entire central nervous system. The photograph shows a mouse injected systemically with trypan blue (a dye that adheres to plasma proteins) and dissected from the dorsal side to reveal the central nervous system. Note that all structures throughout the body, except the central nervous system, take up the dye. Milton W Brightman and Thomas S Reese of the National Institute of Neurological Disorders and Stroke prepared the image, which was kindly provided by MW Brightman.

(16). This is vital for those reactions that rely on NADPH (eg, isocitrate dehydrogenase) rather than NADH because it is NADH that is oxidized by the cytochrome system to produce energy.

Glutamate also serves as an important potential fuel reserve. The oxidation of glutamate to oxaloacetate yields 12 ATP per molecule of glutamate. Therefore, when the brain has insufficient glucose concentrations or glycolytic flux is reduced, the brain mobilizes glutamate as a fuel (17). In this regard, the energy available from glutamate is similar to glucose as a fuel reserve.

Compartmentalization of glutamate

In the brain, glutamate exists as a free amino acid divided between 2 separate metabolic compartments located in astrocytes and neurons. These compartments were first recognized in the brain on the basis of radioisotope precursor-product relations

between glutamine and glutamate. To be incorporated into these amino acids from an oxidizable substrate, a label must first be converted into acetyl-coenzyme A (or succinyl-coenzyme A in the case of propionate) and then further oxidized in the Krebs cycle to α -ketoglutarate before exchanging with glutamate. Therefore, the specific radioactivity of glutamate is expected to exceed that of glutamine, as is the case with many substrates such as glucose, ketone bodies, lactate, and glycerol (18). However, several other oxidizable substrates, including some amino acids as well as short- to medium-chain-length fatty acids, label glutamine to a greater specific activity than glutamate (18–22). These results can be explained by the existence of ≥ 2 separate pools of Krebs cycle intermediates, one containing a large pool of glutamate with relatively little conversion to glutamine and the other containing a small glutamate pool that is rapidly metabolized to glutamine. On the basis of considerable evidence, these major pools can be assigned to the neurons and glial cells, respectively (19, 23–26). In support of this concept, compartmentation is almost absent at birth and develops in parallel with glial cells (26), and glutamine synthetase is found only in astrocytes (25).

Neuronal glutamate is contained in ≥ 2 pools, 1 composed of neuronal perikarya and dendrites and the 1 of nerve terminals (vesicles) (26). Nerve impulses trigger the release of glutamate from the presynaptic cell, which in turn binds to the glutamate receptors on the opposing postsynaptic cell. Neurotransmission is terminated by astrocytes and neurons that take up glutamate. Very little glutamate is believed to diffuse away from the synapse.

Excitotoxicity hypothesis of neuronal death

Early studies that used very high doses of glutamate, which were administered systemically, revealed brain damage in areas of the brain that were not protected by the BBB (27, 28). These studies led to the concept that neuronal death could be produced by overstimulation of excitatory amino acid receptors (29–31). Subsequently, this hypothesis became a popular explanation of the pathogenesis of neuronal death in a variety of acute conditions. However, in such cases, the source of glutamate arises from within the brain. For example, during an ischemic episode, release of glutamate (32, 33) from brain cells may result in an

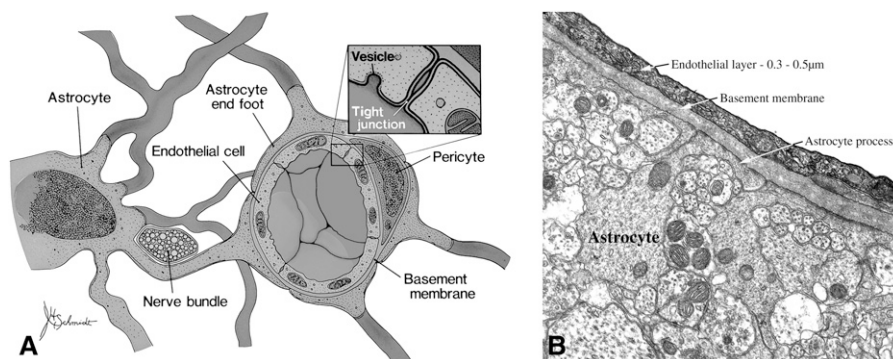


FIGURE 2. Diagram of the blood-brain barrier. A: The blood-brain barrier exists at the level of the endothelial cells of cerebral capillaries. The endothelial cells are joined together by an extensive network of tight junctions. A basement membrane, within which pericytes reside, surrounds the endothelial cells as does a layer composed of astrocyte processes (so-called end feet). The pericytes are numerous and most likely function as phagocytes. The astrocyte layer serves as a metabolic barrier. For example, astrocytes incorporate NH_4^+ into glutamine and metabolize short-chain fatty acids. B: An electron micrograph of a cerebral capillary shows the basic elements (provided through the courtesy of Robert Page, Pennsylvania State University College of Medicine). Reproduced with permission from reference 1.

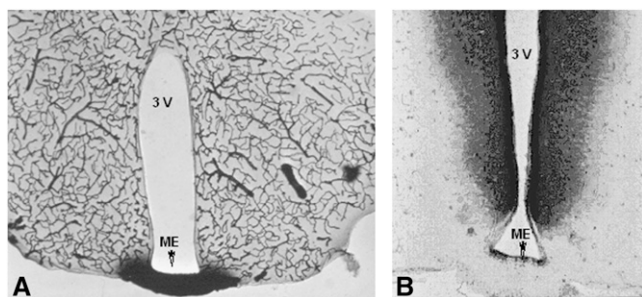


FIGURE 3. A: Horseradish peroxidase injected into the general circulation. Note that the enzyme is restricted to capillaries except in the median eminence (ME), which has fenestrated capillaries. No horseradish peroxidase reaches any of the ventricles, including the third ventricle (3V). B: Horseradish peroxidase injected into the 3V. Note that the peroxidase readily penetrates the brain parenchyma, but not the area of the ME. It may be concluded that there is a barrier at the level of the epithelial lining of the ventricle that prevents leakage of metabolites into the ventricles. Milton W Brightman and Thomas S Reese of the National Institute of Neurological Disorders and Stroke prepared the image, which was kindly provided by MW Brightman.

excessive concentration of glutamate in the extracellular fluid (ECF) (34, 35). The extreme excitation of neurons by glutamate in turn may 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, which activate a host of catabolic enzymes, may ultimately result in neuronal death (36). However, under normal conditions, plasma glutamate concentrations are stable and do not change appreciably unless raised by artificial means.

Glutamate in circulation

Plasma glutamate concentrations are in the range of 50–100 $\mu\text{mol/L}$ in humans and other species (37). Even when relatively large quantities of monosodium glutamate have been added to the food of mice, monkeys, or humans, only very small changes in the plasma concentration of glutamate occur (38–41). This is because the intestinal mucosa preferentially metabolizes luminal glutamate and glutamine and uses their carbon skeletons as a source of energy (42, 43). Glutamine is also the primary amino acid by which nitrogen is carried from peripheral tissues to the splanchnic organs. Glutamine is deaminated by phosphate-dependent glutaminases in the intestinal mucosa to glutamate, which is then metabolized completely to produce energy. This process raises the ammonia in the hepatic portal vein to the degree that is optimal for the synthesis of urea (44).

FACILITATIVE AND ACTIVE TRANSPORT SYSTEMS FOR GLUTAMATE IN THE BBB

Early studies of the BBB that used whole-brain perfusions or animals in vivo identified facilitative transporters in the BBB membrane that are saturable and stereoselective (45–47). Because the substrate was presented to the capillary lumen, it was 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 material quantities, except in the circumventricular organs (48–50). Until recently, this has been a conundrum. Why should there be

a transport system for an amino acid that is synthesized within the brain in large quantities? It required a different approach to the study of the BBB and new techniques to provide an explanation.

Studying each side of the BBB separately

The earlier models for studying the BBB in vivo gave incomplete information because metabolites have to pass both the luminal and the abluminal membranes to gain access to brain cells. In our laboratory, we studied both sides of the BBB by making use of isolated luminal and abluminal membranes obtained from fresh bovine brain.

The respective plasma membrane domain, when separated by the procedure developed by Betz et al (9, 10), demonstrated differences between the 2 sides of the BBB (polarity). We realized that both the luminal and the abluminal membranes form sealed spherical vesicles that are, for the most part, right side out and suitable for the study of transport in vitro (51, 52). The isolated membrane vesicles maintain their transport properties, and therefore they were used to characterize the contribution of each membrane domain to BBB activity. We found facilitative carriers for glutamate to exist exclusively in the luminal membranes and energy-dependent Na^+ -cotransporters in the abluminal membrane.

Facilitative transport of glutamate in the luminal membrane

Lee et al (53) measured facilitative glutamate transport separately in luminal and abluminal membranes and found that facilitative glutamate transport exists only in the luminal border in a position to allow the release of glutamate from endothelial cells to the plasma. Luminal carriers of amino acids have no dependence on Na^+ gradients (46, 54–58) and are therefore energy independent. Three broad classes of facilitative carriers exist: large neutral amino acids, cationic amino acids, and acidic amino acids, and each transports several amino acids (59). As mentioned, the presence of a transporter for acidic amino acids with a high affinity and a low capacity (12, 60, 61) was an enigma for many years because both glutamate and aspartate are nonessential amino acids that are synthesized and accumulated in high concentrations in the brain.

Active transport systems expel glutamate from the ECF

Ordinarily, ECF glutamate is kept very low ($\approx 0.5\text{--}2 \mu\text{mol/L}$) (61). In fact, the concentration of glutamate and aspartate in cerebrospinal fluid is lower than that of any other amino acid group (Figure 4). The large gradient between brain cells and ECF is maintained by a family of Na^+ -dependent glutamate transporters known as excitatory amino acid transporters (EAATs). These transporters couple the steep Na^+ gradient that normally exists between the ECF and brain cells. Currently, 5 members of the EAAT family have been identified (62, 63). They reside in the plasma membranes of astrocytes (35, 62, 64, 65), neurons (62, 66–70), and the BBB (71). The Na^+ -dependent transporters work at the limit of their ability to maintain the glutamate gradient between the brain cells and the ECF and, of course, the steep Na^+ gradient as well (extracellular \gg intracellular) that is maintained by Na^+/K^+ -ATPase. If the oxygen supply is insufficient to maintain ATP concentrations, membrane Na^+/K^+

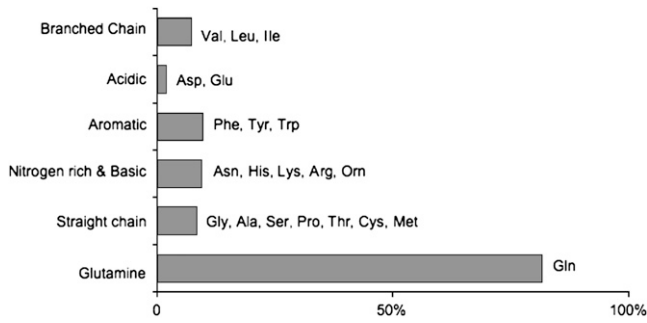


FIGURE 4. Extracellular fluid (ECF) concentrations of amino acid groups compared with plasma. Note that the excitatory amino acids glutamate and aspartate are almost undetectable. Glutamine, which is believed to be nontoxic, has similar concentrations in the plasma and the ECF. Reproduced with permission from reference 1.

K^+ -ATPase ceases 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.

Of the 5 known Na^+ -dependent glutamate transporters (EAATs 1–5) (72), ≥ 3 exist in the abluminal membrane of the BBB (71, 73). They are voltage dependent and collectively have an apparent K_m of $14 \mu\text{mol/L}$ at a transmembrane potential of -61 mV (53, 71). Western blot analysis confirmed that the glutamate transporters are present exclusively in the abluminal membranes; no EAATs were detectable in luminal membranes (71). Collectively, the EAAT family is the most powerful of the Na^+ -dependent amino acid transporters found in the abluminal membrane to date (1).

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 pumps 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 into the brain is slow or almost undetectable (48–50, 61). Glutamate may enter the

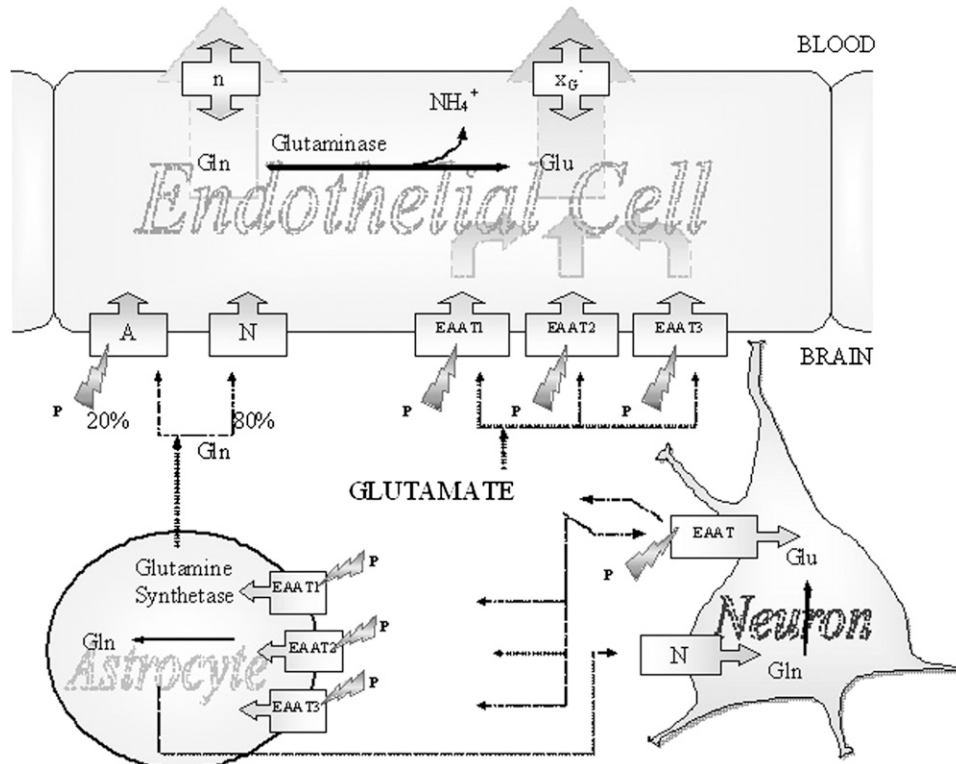


FIGURE 5. Glutamate and glutamine transport between neurons, astrocytes, and endothelial cells of the blood-brain barrier. Glutamate is the most abundant excitatory neurotransmitter in the mammalian nervous system. At chemical synapses, glutamate is stored in vesicles. Nerve impulses trigger the release of glutamate from the presynaptic cell. Na^+ -dependent glutamate transporters (excitatory amino acid transporters; EAATs) are found in neuronal and glial membranes. These transporters play the important role of regulating concentrations of glutamate in the extracellular space, which maintains low concentrations. After glutamate is released as the result of an action potential, glutamate transporters quickly remove it from the extracellular space and thereby terminate the synaptic transmission. Without the activity of glutamate transporters, glutamate would build up and kill cells in a process called excitotoxicity, in which excessive amounts of glutamate act as a toxin to neurons. The activity of glutamate transporters also allows glutamate to be recycled. In cases of brain injury or oxygen insufficiency, the EAATs can work in reverse, and excess glutamate can accumulate outside cells, which rapidly halts neurotransmission. At least 3 EAATs are present in the abluminal membrane of the blood-brain barrier. These EAATs move glutamate into the endothelial cells from which egress is possible through the facilitative transporters in the luminal membrane. There are transporters capable of pumping glutamine from the extracellular fluid into endothelial cells, and glutaminase within endothelial cells may also hydrolyze glutamine to glutamate and NH_4^+ . No carrier is necessary for NH_4^+ , which may diffuse as NH_3 . A, Na^+ -dependent system A; N, Na^+ -dependent system N; XG^- , facilitative glutamate transporter; n, facilitative glutamine transporter. The lightning symbols indicate Na^+ dependence, and P indicates stimulation by pyroglutamate. Reproduced with permission from reference 1.

endothelial cells, but net movement of glutamate from endothelial cells to the brain is almost 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.

GLUTAMATE, GLUTAMINE, AND AMMONIA REMOVAL

The description of the organization of the BBB also provides an explanation for a long-standing puzzle regarding brain NH_4^+ metabolism. Various measurements have shown that 20–50% of the NH_4^+ circulating through the blood vessels in the brain passes the BBB and is incorporated into the amide group of glutamine by astrocytes (23, 24). It is curious, however, that it has not been possible to consistently measure arteriovenous differences of NH_4^+ (24). If there were no mechanism for the removal of glutamine, it would accumulate in brain, thereby raising the osmolarity and causing swelling. The situation is now clearer. Glutamine and glutamate are pumped from the ECF into endothelial cells. Glutamine is at least partially metabolized to NH_4^+ and glutamate. The remaining glutamine as well as NH_4^+ and glutamate are free to diffuse across the luminal membrane into the blood (53, 71). Thus the rate of NH_4^+ uptake and release are balanced.

This new knowledge also explains how the entry of glutamine and glutamate into the central nervous system is restricted even though carrier activities for both amino acids have been described (49, 59, 74). Glutamine and glutamate can traverse the luminal membrane in 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 3 Na^+ -dependent carriers in the abluminal membrane that are driven by the steep Na^+ gradient that exists between the 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 brain but also to actively export these amino acids and 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^+ .

γ -Glutamyl cycle and the transport of amino acids across the BBB: the role of pyroglutamate in regulation

Meister et al (75, 76) noted that γ -glutamyl transpeptidase is present in tissues that were believed to actively transport amino acids. Such tissues include the brush border of the proximal convoluted tubules of the kidney (77), lactating mammary glands (78), the apical portion of the intestinal epithelium (79), the choroid plexus (80), and the BBB (81). However, the BBB differs from most tissues that actively transport amino acids. First, the BBB is composed of endothelial, not epithelial, cells. Second, there are no Na^+ -dependent amino acid transporters on the luminal surface of the BBB. Therefore, the BBB is not associated with energy-dependent amino acid uptake from plasma. Instead, the organization of the BBB provides a mechanism for

active removal of glutamate, glutamine, and other amino acids. It has therefore been puzzling that brain capillaries have such high γ -glutamyl transpeptidase activity. γ -Glutamyl transpeptidase is the first enzyme in the γ -glutamyl cycle that produces pyroglutamate (pGlu) within the cell (82, 83) (also known as oxoproline—the cyclized amide of glutamate). pGlu can stimulate Na^+ -dependent glutamate transport at the BBB. It has been suggested that the generation of pGlu is part of a control mechanism that influences the concentration of glutamate in the brain ECF. This activity may be part of a short-term regulatory mechanism by which the removal of this potentially deleterious glutamate is accelerated. Furthermore, it suggests that EAAT transporters in the BBB may serve as a therapeutic target in circumstances where glutamate cytotoxicity occurs, eg, with ischemia or brain injury. Stimulation of EAAT transporters by pGlu or comparable molecules during reperfusion may accelerate the restoration of glutamate homeostasis and reduce the development of cerebral infarcts.

Diabetes does not affect glutamate transport at the BBB

A recent conference report on the use of glutamate in food suggested that, although addition of glutamate salts to foods may be considered harmless to the general population, this may not be the case in individuals in whom the BBB may be impaired, such as in patients with diabetes (84). We studied the possibility that the BBB permeability of glutamate is changed by diabetes in 2 rat models: 1) a model of diet-induced obesity that causes insulin resistance (85, 86), a mild form of diabetes, and 2) a model of a more severe form of diabetes, induced by streptozotocin, which destroys most of the pancreatic β cells and causes chronic hyperglycemia and ketonemia. Both forms showed no change in the BBB; that is, there was no evidence of increased permeability (RA Hawkins, A Mokashi, J Viña, and J Fernstrom, unpublished observations, 2008).

CONCLUSIONS

The current concept of the BBB is that cerebral endothelial cells are not simply passive barriers; rather, they participate actively in regulating the composition of the brain ECF. The abluminal and luminal membranes seem to be working in a complementary fashion with, for the most part, active transport occurring at the abluminal membrane and facilitative transport at the luminal membrane. The abluminal membrane is in direct contact with the ECF and has Na^+ -dependent transport systems and a Na^+ gradient that can move metabolites out of the ECF against a concentration gradient. The luminal membrane primarily has facilitative transport systems that allow molecules to enter and exit the endothelial cells. (Other articles in this supplement to the Journal include references 87–115.)

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