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Published in:
Glial Amino Acid Transporters

DOI:
10.1007/978-3-319-55769-4_15

Publication date:
2017

Document version
Final published version

Citation for pulished version (APA):
Helms, H. C. C., Nielsen, C. U., Waagepetersen, H. S., & Brodin, B. (2017). Glutamate Transporters in the Blood-Brain Barrier. In A. Ortega, & A. Schousboe (Eds.), *Glial Amino Acid Transporters* (1 ed., pp. 297-314). Springer. Advances in Neurobiology, No. 1, Vol.. 16 https://doi.org/10.1007/978-3-319-55769-4_15

Glutamate Transporters in the Blood-Brain Barrier

Hans Christian Cederberg Helms, Carsten Uhd Nielsen, Helle Sønderby Waagepetersen, and Birger Brodin

Abstract The amino acid L-glutamate serves a number of roles in the central nervous system, being an excitatory neurotransmitter, metabolite, and building block in protein synthesis. During pathophysiological events, where L-glutamate homeostasis cannot be maintained, the increased brain interstitial fluid concentration of L-glutamate causes excitotoxicity. A tight control of the brain interstitial fluid L-glutamate levels is therefore imperative, in order to maintain optimal neurotransmission and to avoid such excitotoxicity. The blood-brain barrier, i.e., the endothelial lining of the brain capillaries, regulates the exchange of nutrients, gases, and metabolic waste products between plasma and brain interstitial fluid. It has been suggested that brain capillary endothelial cells could play an important role in L-glutamate homeostasis by mediating brain-to-blood L-glutamate efflux. Both in vitro and in vivo studies have demonstrated blood-to-brain transport of L-glutamate, at least during pathological events. A number of studies have shown that brain endothelial cells express excitatory amino acid transporters, which may account for abluminal concentrative uptake of L-glutamate into the capillary endothelial cells. The mechanisms underlying transendothelial L-glutamate transport are however still not well understood. The present chapter summarizes the current knowledge on blood-brain barrier L-glutamate transporters and the suggested pathways for the brain-to-blood L-glutamate efflux.

Keywords Excitotoxicity • EAAT • Neurovascular unit • Brain glutamate efflux • Glutamate metabolism

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A. Ortega, A. Schousboe (eds.), *Glial Amino Acid Transporters*, Advances in Neurobiology 16, DOI 10.1007/978-3-319-55769-4_15

1 Introduction

L-glutamate is one of the most common amino acids, a building block in protein synthesis and an important metabolite in intermediary metabolism linking carbohydrate and amino acid metabolism. L-glutamate is also considered to be the most abundant excitatory neurotransmitter in the vertebrate brain (Zhou and Danbolt 2014). L-glutamate is stored in vesicles in the synapses of glutamatergic neurons and released into the synaptic cleft upon nerve stimulation. The transmitter is rapidly removed from the synaptic cleft and the brain interstitial fluid (ISF) by the concerted actions of glutamate transporters in neurons and astrocytes. Prolonged elevated concentrations of L-glutamate in the ISF are highly cytotoxic since continued and excessive stimulation of glutamate receptors will cause abnormal high intracellular calcium levels in neurons, followed by activation of a number of enzyme cascades and, eventually, cell death. This phenomenon has been termed excitotoxicity (Nakanishi 1992; Rothman 1984) and occurs during a number of pathophysiological conditions (Gillessen et al. 2002; Leibowitz et al. 2012).

The blood-brain barrier, i.e., the endothelium of the brain capillaries, acts as a gatekeeper between the blood and the brain ISF. One major role of the brain endothelium is to separate the blood plasma constituents and the ISF, since the blood concentration of L-glutamate is 10–100-fold higher than in the brain, and free passage across the barrier would cause excitotoxicity. A number of studies have indicated that the brain endothelium might also play an active role in the regulation of brain L-glutamate concentrations beyond just being a static barrier to the peripheral circulation. Brain endothelial cells express excitatory amino acid transporters (EAAT's) and have been proposed to efflux L-glutamate via the concerted actions of abluminal (brain-facing) and luminal (blood-facing) transport proteins (Boyko et al. 2014; Roberts et al. 2014; Teichberg et al. 2009; Campos et al. 2011a; Cohen-Kashi-Malina et al. 2012; Helms et al. 2012; Lyck et al. 2009).

Previous reviews of the field have focused on the role of possible blood-to-brain transport of L-glutamate originating from dietary monosodium glutamate (Hawkins 2009), on EAATs (Danbolt et al. 2016) and blood L-glutamate scavenging (Teichberg et al. 2009), on possible therapeutic applications of blood glutamate scavenging (Castillo et al. 2016) and on potential clinical relevance of BBB-glutamate efflux (Cederberg et al. 2014). The aim of the present chapter is to supply an overview of L-glutamate transport proteins in the brain capillary endothelium, as well as their role in uptake and transendothelial transport. Possible metabolism of glutamate in the endothelial cells and astrocytes will be discussed. Finally, we will address the proposed concept of “glutamate scavenging” and point toward areas which in our point of view warrants further investigations.

2 Early Studies on Glutamate Transport Across Brain Endothelium

There is a large difference between L-glutamate concentrations in blood and in brain tissue interstitial fluid. Free plasma concentrations have been estimated to be in the range of 30–90 μM (Hagenfeldt and Arvidsson 1980; Graham et al. 1995), whereas erythrocyte concentrations are in the range of 280 μM (Hagenfeldt and Arvidsson 1980) to 450 μM (Divino Filho et al. 1997), thus giving whole blood concentrations of L-glutamate at approximately 140 μM taking the volume fractions into consideration (Hagenfeldt and Arvidsson 1980).

Brain ISF L-glutamate concentrations are in the lower μmolar range. Sampling and analysis of brain ISF from the resting undisturbed brain is not trivial, but microdialysis studies have indicated values of 0.1–3 μM (Persson and Hillered 1992). Estimations of L-glutamate concentrations in the cerebrospinal fluid yield values in a similar range, i.e., 0.5–3 μM (Iijima et al. 1978; Spink et al. 1986).

The large concentration difference of L-glutamate across the blood-brain barrier could indicate that the brain endothelial cells have a very low permeability for L-glutamate, an active transport of L-glutamate from brain ISF to blood, a fast metabolism of L-glutamate, or a combination of these, along with a fast metabolism/clearance of L-glutamate in the ISF by astrocytes and neurons (Schousboe et al. 2014; Waagepetersen et al. 2005).

The concentrations of L-glutamate in brain endothelial cells have been estimated to be approximately 750 nmol g^{-1} (Cardelli-Cangiano et al. 1981), equivalent to approximately 785 μM (assuming an endothelial cell density of 1.048 g ml^{-1} (Pries et al. 2000)). Considering that the free L-glutamate concentration is ~60 μM in the blood, the concentration gradient will favor transport from the cell cytosol to the blood and not favor uptake from blood to cell. Early studies on blood-to-brain transport of tracer amounts of radiolabeled glutamate in rats showed a low brain uptake (Oldendorf 1971; Sershen and Lajtha 1976), as measured by the brain uptake index (BUI) method. The uptake of radiolabeled tracer was shown to be inhibited by excess non-labeled L-glutamate, indicating that the transport from blood to brain took place via a saturable carrier-type mechanism (Oldendorf and Szabo 1976). These estimates were, however, based on unidirectional influx values.

Despite the cell-to-blood concentration gradient of L-glutamate, there would still exist a unidirectional isotope flux in the blood-to-cell direction, as observed by Oldendorf and Szabo, but not a net uptake (Oldendorf and Szabo 1976). In summary, the initial studies indicated the presence of a carrier mechanism for L-glutamate in the luminal membrane of the brain endothelium but also a large gradient for L-glutamate transport from plasma to cell, indicating either that the observed fluxes only were indicative of unidirectional influx (plasma to cell) in a situation where a net efflux was occurring or that the thermodynamically "uphill" transport of L-glutamate from plasma to cell was coupled to a dissipation of a concentration gradient (either a co- or counter transport) or a pump-type transporter (driven by hydrolysis of ATP). This issue was addressed by Drewes and colleagues,

who showed a net efflux of L-glutamate from the brain to the blood in studies on perfused dog brains (Drewes et al. 1977). In brief, the author's measured concentrations of amino acids in the arterial and venous perfusates using chromatography techniques, and net movements of amino acids, were calculated from the concentration differences in arterial and venous concentrations. They observed a net efflux of L-glutamate from the brain in the order of ~1 μmol 100 g brain^{-1} min^{-1} under basal conditions (Drewes et al. 1977). On the basis of these data, Pardridge suggested that an active L-glutamate brain-to-blood efflux system was present and functional under resting conditions in the brain capillary endothelium (Pardridge 1979). Hutchison and colleagues investigated uptake kinetics of L-glutamate into isolated rat brain capillaries (Hutchison et al. 1985). They demonstrated that a high-affinity (K_M of approximately 2 μM), temperature-dependent, and ouabain-sensitive L-glutamate uptake system was present in the capillaries, which could account for the previously observed brain efflux. This was further supported by Hosoya et al., who performed intracerebral microinjections of radioactive L-glutamate, L-aspartate, and D-aspartate in rats (Hosoya et al. 1999). They demonstrated rapid clearance from the brain of L-glutamate and L-aspartate, which correlated with the appearance of the two isotopes in jugular vein samples, whereas D-aspartate stayed in the brain compartment. Hosoya et al. used thin layer chromatography to verify that at least the main part of the appearing radioactivity in the blood originated from intact L-aspartate/L-glutamate (approximately 70% and 84%, respectively) (Hosoya et al. 1999). Initially, this efflux was not believed to be associated with EAAT transporters, partly because of lack of D-aspartate efflux and partly because immunolabeling studies had not shown EAAT1 and EAAT2 expression in rat brain endothelial cells (Hosoya et al. 1999; Chaudhry et al. 1995; Lehre et al. 1995). However, in the recent decades, evidence has accumulated that EAATs are present in brain capillary endothelial cells and may take part in the brain L-glutamate efflux (see sections below).

3 Expression of Glutamate Transporters in the Brain Capillary Endothelium

3.1 Transporters of Glutamate

Glutamate transporters are transmembrane proteins belonging to the solute carrier (SLC) family 1, SLC 7, SLC 17, or SLC25 (see Table 1) (Fotiadis et al. 2013; Kanai et al. 2013; Palmieri 2013; Reimer 2013; Hegedus and Taale 2013). The SLC1 family consists of seven members, with five members being excitatory amino acid transporters (EAATs) transporting glutamate and aspartate and two members being alanine, serine, and cystine (ASC) transporters transporting alanine, serine, cystine, and threonine (see Table 1 (Kanai et al. 2013; Hegedus and Taale 2013)). The K_m values described for L-glutamate transport by EAAT1 are in the range of 7–20 μM;

Table 1 Transporters of glutamate

Gene	Protein	Main cell types in the CNS	Cellular localization	References
SLC1A1	EAAT3 (EAAC1)	Neurons and substantia nigra, red nucleus, hippocampus, cerebral cortical layers	Plasma membrane of postsynaptic neurons	Shashidharan et al. (1994), Kanai and Hediger (1992), Kanai et al. (1994), Rothstein et al. (1994)
SLC1A2	EAAT2 (GLT-1)	Astrocytes Glia cells	Plasma membrane	Pines et al. (1992), Danbolt et al. (1992)
SLC1A3	EAAT1 (GLAST)	Glia cells Astrocytes	Plasma membrane	Storck et al. (1992), Danbolt et al. (1994)
SLC1A6	EAAT4	Purkinje cell somas and dendrites	Plasma membrane	Fairman et al. (1995), Lin et al. (1998)
SLC1A7	EAAT5	Retina	Plasma membrane	Arriza et al. (1997)
SLC7A11	xCT (associate with 4F2hc to form system x_c^-)	Brain (neurons, astrocytes, and glial cells) and spinal cord	Plasma membrane	Kim et al. (2001), Jackman et al. (2010), Fogal et al. (2007)
SLC7A13	AGT-1 (associate with rBAT as heavy chain)	Not expressed	Basolateral plasma membrane in proximal straight tubules and distal convoluted tubules (Matsuo et al. 2002) but apical in another study (Nagamori et al. 2016)	Blondeau (2002), Matsuo et al. (2002)
SLC17A6	VGLUT2	Small: (0–500 μm^2) Medium: (500–1300 μm^2) DRG	Vesicular membrane	Malet et al. (2013), Aihara et al. (2000)
SLC17A7	VGLUT1	Medium: (500–1300 μm^2) Large: (1300–2900 μm^2) DRG	Vesicular membrane	Malet et al. (2013), Ni et al. (1994)
SLC17A8	VGLUT3	Small: (0–500 μm^2) Medium: (500–1300 μm^2) DRG	Vesicular membrane	Malet et al. (2013), Takamori et al. (2002)
SLC25A12	AGC1 (aralar)	Neurons in the brain stem and spinal cord	Mitochondrion inner membrane	del Arco and Satrustegui (1998), Ramos et al. (2003)

(continued)

Table 1 (continued)

Gene	Protein	Main cell types in the CNS	Cellular localization	References
SLC25A13	AGC2 (citrin)	Limited to neuronal cluster	Mitochondrion inner membrane	Kobayashi et al. (1999), Del Arco et al. (2000), Contreras et al. (2010)
SLC25A18	GC2	Expressed in brain panels	Mitochondrion inner membrane	Fiermonte et al. (2002)
SLC25A22	GC1	Expressed in brain panels, and in developing human nervous system, notably in the hippocampus, cortex, and brainstem	Mitochondrion inner membrane	Fiermonte et al. (2002), Molinari et al. (2005)

DRG dorsal root ganglia

for EAAT2 and EAAT3, they are 12–18 μM and 8–30 μM, respectively. EAAT4 has the highest affinity reported for glutamate transport via EAATs with K_m values in the range of 0.6–3.3 μM, while EAAT5 has the lowest affinity with K_m values in the range of 61–63 μM (for references see (Danbolt et al. 2016)). The uptake of L-glutamate via EAATs is indirectly dependent on the energy status of the cell since the translocation cycle includes cotransport of one molecule of L-glutamate with three sodium ions and one hydrogen ion and an exchange with one potassium ion (Levy et al. 1998; Zerangue and Kavanaugh 1996). The transport activity and direction is thus coupled to the sodium-potassium ATPase (Rose et al. 2009). SLC7 is the family of cationic amino acid transporters and glycoprotein-associated proteins. SLC7A11 is the light chain that coupled with the heavy chain 4F2hc forms system x_c^- that is a cystine/L-glutamate exchanger and SLC7A13 which associate with rBAT as heavy chain forming an L-aspartate/L-glutamate exchanger, apparently not expressed in the brain (SLC tables). The SLC 17 family contains vesicular glutamate transporters which are involved in glutamate accumulation in membrane vesicles in presynaptic neurons. These transporters are electrogenic chloride-dependent glutamate transporters. The SLC25 family contains mitochondrial glutamate transporters. SLC25A12 and SLC25A13 are calcium- and proton-dependent exchangers of cytoplasmic glutamate with mitochondrial aspartate which takes place across the inner mitochondrial membrane. SLC25A18 and SLC25A22 are proton-coupled glutamate transporters involved in glutamate transport across the inner mitochondrial membrane. In the context of glutamate transport across the brain endothelium, EAAT transporters are relevant to consider (Table 2), acknowledging that both VGLUT and EAATs are important for overall glutamate levels in the healthy brain and in disease.

Table 2 Overview of EAAT subtype expression patterns in capillaries and capillary-derived cell cultures from different species

		EAAT1	EAAT2	EAAT3	References
Capillaries or intact tissue preparations	mRNA	B,H, M	B, M	M	Helms et al. (2012), Lyck et al. (2009), Daneman et al. (2010), Guo et al. (2012), O'Kane et al. (1999), Shawahna et al. (2011)
	Protein	B, H, M, Ma	M	M	Roberts et al. (2014), Helms et al. (2012), Chun et al. (2011), Uchida et al. (2011), Lecointre et al. (2014), O'Kane et al. (1999), Hoshi et al. (2013)
Cell culture	mRNA	B		B, M	Helms et al. (2012), Lyck et al. (2009)
	Protein	B, M, P, R	M, P	B, M, P, R	Campos et al. (2011a), Cohen-Kashi-Malina et al. (2012), Helms et al. (2012), Lecointre et al. (2014)

B bovine, *H* human, *M* mouse, *P* porcine, *R* rat, *Ma* marmoset

3.2 Expression of EAATs in Intact or Isolated Brain Capillaries

Chun and co-workers performed a proteomics study on freshly isolated mouse brain microvessel membranes, and, using multidimensional protein identification technology, they identified 1143 proteins of which 101 were membrane transporters of mainly the SLC family (Chun et al. 2011). The gene products of plasma membrane glutamate transporters Slc1a2 and Slc1a3 were among the most abundant transporters, but also mitochondrial glutamate transporters from Slc25a22 and Slc25a18 genes were found (Chun et al. 2011). Gene products of Slc17a7 and Slc1a1 were also identified with spectral counts above 5 (Chun et al. 2011). In contrast, Ushida and co-worker found 20 membrane transporters in human brain microvessels and found only EAAT1 and not EAAT3 protein amounts above the detection limit (Uchida et al. 2011). In brain capillaries isolated from marmoset brain, EAAT1 protein was found highly expressed (Hoshi et al. 2013). Moreover, a recent electron microscopy study by Roberts et al. showed a clear EAAT1 expression in brain capillary endothelial cells from human postmortem cortex samples (Roberts et al. 2014). Brain capillaries from mice have been shown to have a high expression of *EAAT3 mRNA* (Lyck et al. 2009; Daneman et al. 2010; Guo et al. 2012) as well as protein expression of EAAT1, EAAT2 and EAAT3 (although mainly subtypes 2 and 3) (Lecointre et al. 2014). Freshly isolated bovine brain capillaries express *EAAT1*, *EAAT2*, and *EAAT3 mRNA*, whereas only EAAT1 has been detected at the protein level (Helms et al. 2012; O'Kane et al. 1999). Large quantities of *EAAT1 mRNA* (Shawahna et al. 2011) were found in human brain capillaries. These studies indicate that EAATs are present in endothelial cells, even though a study on rat

capillaries revealed little or no EAAT staining on the abluminal surface of brain capillaries (Chaudry et al. 1995). However the subtype specific expression pattern may vary between species. It has to be kept in mind that contamination by glial tissue or remnants of astrocyte end feet may be a general concern as protein fragments from these tissues will greatly affect conclusions made.

3.3 Expression and Functional Activity of Excitatory Amino Acid Transporters in Cultured Endothelial Cells and Vesicle Preparations

Abluminal sodium-dependent saturable uptake was initially demonstrated in membrane vesicles from bovine brain endothelial cells (Lee et al. 1998). The uptake was attributed to EAATs through protein expression and uptake studies. The studies demonstrated expression of EAAT1, EAAT2, and EAAT3 in abluminal membrane vesicles as well as high-affinity uptake matching that of EAATs in other cell types (O'Kane et al. 1999). In cell cultures, EAAT3 seemed to be the main transporter in mouse brain endothelial cells (Lyck et al. 2009; Lecointre et al. 2014), whereas EAAT1 displayed the highest expression level in bovine endothelial cells co-cultured with rat astrocytes (Helms et al. 2012). Helms et al. showed that EAAT1 is the dominant abluminal L-glutamate transporter in this model (Helms et al. 2016). EAAT1, EAAT2, and EAAT3 were all present in cultured endothelial cells from rat and porcine brains although EAAT3 seemed to be expressed at a higher level than the other subtypes (Campos et al. 2011a; Cohen-Kashi-Malina et al. 2012).

3.4 The Luminal Glutamate Transport System

Early in vivo studies indicate the presence of a facilitative L-glutamate transporter at the luminal membrane of the endothelial cells. Uptake of L-glutamate from the blood circulation and into the brain was shown in rats after a carotid bolus injection of radiolabeled L-glutamate (Oldendorf and Szabo 1976). The putative transporter was named x_G^- (Christensen 1984) and was shown to be independent of sodium and inhibited by L-glutamate and L-aspartate (Oldendorf and Szabo 1976; Lee et al. 1998; Benrabh and Lefauconnier 1996). The transporter has not been cloned, and it is possible that an already known amino acid transporter facilitates the passage of the luminal membrane. The glutamate-cystine exchanger (x_c) could be a candidate to a transporter which could efflux intracellular L-glutamate from the endothelial cells to the blood in exchange for cystine uptake. However, previous studies have shown no effects of cystine on luminal endothelial L-glutamate uptake (O'Kane et al. 1999; Benrabh and Lefauconnier 1996). In the mouse brain endothelial cell line, MBEC4A, *xCT*, and *4F2hc mRNA* are expressed, and carrier-mediated cystine and glutamate uptake has been measured, although it may be difficult to distinguish

luminal and abluminal uptake in cells cultured on the bottom of cell culture wells (Hosoya et al. 2002). In rat brain slices, a low-affinity L-glutamate transporter has been described (Balcar and Johnston 1972; Benjamin and Quastel 1976). This transporter of L-glutamate was inhibited by L-glutamate, L-aspartate, and L-homocysteate (Cox et al. 1977), which matches the inhibition pattern observed for the x_G^- transporter (Benrabh and Lefauconnier 1996). The low-affinity L-glutamate uptake has a K_M of 1–2 mM (Balcar and Johnston 1972; Benjamin and Quastel 1976). This matches the K_M value for L-glutamate in luminal membrane vesicles from bovine brain endothelium, as determined by Lee et al. (1998). Helms et al. found kinetic and inhibition patterns matching the presumed low-affinity glutamate transporter by measuring luminal glutamate uptake in cultured bovine brain endothelial cells (Helms et al. 2016). An experimental limitation to the study of exchanges may be to have appropriate intracellular glutamate levels in intact cell cultures in vitro and to functionally show the exchange mechanism via cystine uptake.

4 Glutamate Metabolism in Brain Endothelial Cells

Efficient uptake of glutamate into astrocytes is followed by synthesis of glutamine catalyzed by the cytosolic enzyme glutamine synthetase. Glutamine is excreted from the astrocytes and known to be taken up by neurons to be reused as precursor for neurotransmitter glutamate as part of the glutamate-glutamine cycle. In addition, L-glutamate is extensively oxidatively metabolized in astrocytes, initially to α-ketoglutarate either via an oxidative deamination catalyzed by glutamate dehydrogenase or an aminotransferase of which aspartate aminotransferase (AAT), alanine aminotransferase (ALAT), and branched chain aminotransferase (BCAT) have the highest activities in the brain (Danbolt 2001). The observed efflux across the blood-brain barrier obtained using radiolabeled L-glutamate could therefore in theory be explained by uptake and metabolism in astrocytes and transport of labeled metabolites across the endothelial cells. Two studies tested the effect of glutamine synthetase inhibition using methionine sulfoximine in endothelial/astrocyte co-cultures and found no effects on the transcellular L-glutamate transport (Cohen-Kashi-Malina et al. 2012; Helms et al. 2012). However, conversion to α-ketoglutarate and further oxidation of the radiolabeled L-glutamate may still result in radiolabeled metabolites, which would influence estimates of the transendothelial transport of L-glutamate, using radiolabel. Astrocytes are known to release lactate as product of glutamate oxidation, via malic enzyme catalyzed oxidation of malate to pyruvate and subsequently operation of lactate dehydrogenase (Olsen and Sonnewald 2015). Another candidate is the TCA cycle intermediate citrate, which is also released from astrocytes (Westergaard et al. 1994). In agreement with this, citrate and lactate formed from exogenously applied glutamate was found in the abluminal medium (where astrocytes are present) of a noncontact co-culture of astrocytes and endothelial cells (Helms et al. 2016).

Alternatively, glutamate may be metabolized in the endothelial cells following EAAT-mediated uptake. Although endothelial cells in general are known to rely

mostly on aerobic glycolytic energy production, Oldendorf et al. demonstrated a high density of mitochondria in brain endothelial cells (Oldendorf et al. 1977). Interestingly, mitochondria and EAAT1 and EAAT2 co-localize in astrocytes, supporting rapid glutamate oxidation and fueling of the energy requiring uptake by glutamate degradation (Pajecka et al. 2015; Robinson and Jackson 2016). It has been suggested that endothelial cells may oxidize L-glutamate to fuel the ATP-binding cassette (ABC) drug efflux transporters (Mann et al. 2003). It was recently shown, using a noncontact co-culture of astrocytes and endothelial cells exposed to ^{13}C glutamate in the abluminal medium, that glutamate is indeed oxidatively metabolized in the endothelial cells (Helms et al. 2016). This was evident from the ^{13}C labeling of glutamate and the TCA cycle intermediates, α-ketoglutarate, succinate, and malate observed in the endothelial compartment. A complete oxidative degradation of glutamate requires an initial oxidation of malate or oxaloacetate to pyruvate either via malic enzyme activity or the concerted action of phosphoenolpyruvate carboxykinase and pyruvate kinase, respectively. Pyruvate is next either reduced to lactate or decarboxylated to acetyl CoA. Lactate is released, whereas acetyl CoA is further processed to CO_2 in the TCA cycle. The study demonstrated formation of pyruvate and further metabolism to lactate and acetyl CoA in the endothelial cells. The relative extent to which glutamate was metabolized to acetyl CoA and further metabolized in the TCA cycle was higher in the endothelial cells compared to the astrocytes. This may at least partly be explained by a low oxidative glucose metabolism in endothelial cells, and it supports an important role of glutamate as an energy substrate for the endothelial cells. The complete oxidation of glutamate is in accordance with the expression of glutamate dehydrogenase in endothelial cells (Zhang et al. 2014). The operation of glutamate dehydrogenase provides a net entrance of α-ketoglutarate to the TCA cycle, in contrast to aminotransferases that involves another keto acid. Citrate, which is formed in the TCA cycle from the condensation of oxaloacetate and acetyl CoA, was released into the luminal medium and is together with lactate products of glutamate oxidation. In addition, a non-negligible amount of aspartate was found in the luminal medium most likely originating from endothelial metabolism. Net formation of aspartate is formed from glutamate via aspartate aminotransferase in combination with several oxidative steps in the TCA cycle converting α-ketoglutarate to oxaloacetate, a process known as "the truncated TCA cycle" (Skytt et al. 2012). The operation of "the truncated TCA cycle" is also in line with a low oxidative glycolytic rate and availability of acetyl CoA in endothelial cells. Thus, metabolism of glutamate in endothelial cells should be taken into account using radiolabeled glutamate to investigate transcellular processes.

As mentioned above, intact labeled L-glutamate was recovered in the blood after intracerebral injections in rats (Hosoya et al. 1999), indicating that some L-glutamate is transported intact through the cells. Alternatively, paracellular leakage of radiolabeled L-glutamate following the injection may add to the explanation. However, the true balance between metabolism and transport of intact L-glutamate has not yet been fully established and warrants further investigations. The possible transport mechanisms are summarized in Fig. 1.

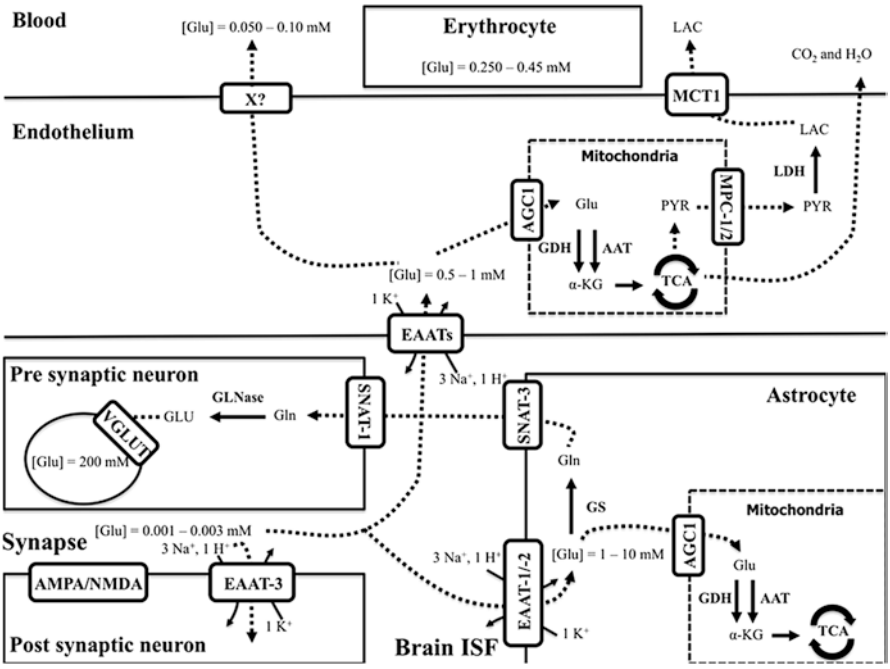

Fig. 1 Glutamate transport and metabolism pathways in the neurovascular unit and how metabolites may appear in the blood after transcellular transport. *α-KG* α-ketoglutarate, *AT* amino acid aminotransferase, *AGC1* mitochondrial aspartate/glutamate carrier-1, *EAAT* excitatory amino acid transporter, *GDH* glutamate dehydrogenase, *GLN* glutamine, *GLNase* glutaminase, *GLU* L-glutamate, *ISF* interstitial fluid, *LAC* lactate, *LDH* lactate dehydrogenase, *MCT-1* monocarboxylic acid transporter-1, *MPC1/2* mitochondrial pyruvate carrier-1/2, *Pyr* pyruvate, *SNAT* small neutral amino acid transporter, *TCA* tricarboxylic acid cycle, *VGLUT* vesicular glutamate transporter, *X?* unknown luminal glutamate transporter (figure modified from (Cederberg et al. 2014) with permission)

5 Blood Glutamate Scavenging: Treatment Paradigm, Clinical Relevance, and Possible Involvement of Endothelial Excitatory Amino Acid Transporters

Based on the observations that L-glutamate can be transported from brain ISF to the blood in animal studies (Drewes et al. 1977; Hosoya et al. 1999) combined with observations of expression of EAATs in brain endothelial cells (O'Kane et al. 1999), it has been proposed that the blood-brain barrier can mediate clearance of L-glutamate and that this might be a mechanism to minimize the excitotoxic damage which may occur under pathophysiological situations. However, the blood glutamate levels of approximately 140 μM (Hagenfeldt and Arvidsson 1980; Graham et al. 1995) limits the blood-brain-barrier-mediated L-glutamate clearance by

providing an unfavorable concentration gradient. The combination of endothelial EAAT expression and the relatively high blood glutamate levels leads to the concept of blood glutamate scavenging, where active lowering of blood glutamate concentrations is hypothesized to increase brain-to-blood transport of L-glutamate because of a more favorable concentration gradient from endothelium to blood (Gottlieb et al. 2003). This was initially demonstrated in rats, where lowering blood glutamate by intravenous injections of oxaloacetate and pyruvate caused lowering of glutamate levels in the cerebrospinal fluid and increased presence of ^{3}H-labeled glutamate in the blood after intracerebroventricular injection (Gottlieb et al. 2003). This experiment demonstrated transport of glutamate from the cerebrospinal fluid to the blood, which is not directly comparable to transport across the blood-brain barrier, but the experiment inspired other investigations of similar concept regarding blood-brain-barrier-mediated efflux.

These experiments have shown beneficial effects of blood glutamate scavenging in rats after middle cerebral artery occlusion, closed head injury, subarachnoid hemorrhage, traumatic brain injury, paraoxon intoxication, and amyloid-beta toxicity (Campos et al. 2011a; Zlotnik et al. 2012; Ruban et al. 2014; Boyko et al. 2012; Perez-Mato et al. 2014; Zlotnik et al. 2009; Zhang et al. 2016) (for review see (Castillo et al. 2016)). Furthermore, blood levels of the endogenous enzyme aspartate aminotransferase have been shown to correlate positively with lower infarct volume and decreased early neurological deterioration (END) after ischemic stroke in humans (Campos et al. 2011b). Scavenging of L-glutamate from the blood circulation seems to be an effective treatment in animal models, and may hold clinical potential, but the exact clearance pathways from the brain to the blood are not yet clearly elucidated. The general assumption is that glutamate is taken up into the brain endothelial cells via EAATs and subsequently transported across the luminal membrane to the blood, when the concentration gradient (cell-to-blood) favors cellular efflux. Breakdown of glutamate in the blood thus creates a more favorable concentration gradient for the glutamate to exit the endothelial cells and thus accelerates the overall clearance from the brain ISF (Castillo et al. 2016). However, an alternative explanation could be that glutamate diffuses across the paracellular space between the brain endothelial cells, which is at least partly opened during stroke, traumatic brain injury, and subarachnoid hemorrhage (Chodobski et al. 2011; Fredriksson et al. 1987; Krueger et al. 2013; Doczi et al. 1986). Diffusion across the paracellular space would also be dependent on the brain-to-blood glutamate concentration gradient, and breakdown of glutamate in the blood would have a similar beneficial effect in this mechanism. Interestingly, a recent study on the effect of blood glutamate scavenging in amyloid-beta toxicity showed that blood glutamate scavenging ameliorates the effects on long-term potentiation of both amyloid-beta and of the EAAT inhibitor, TBOA (Zhang et al. 2016). TBOA has previously been shown to cause neuronal damage by inhibiting glutamate uptake into astrocytes (Montiel et al. 2005), but the fact that this could be ameliorated by blood glutamate scavenging indicates that the brain-to-blood efflux is not dependent on endothelial EAATs exclusively, since these would be inhibited by the TBOA in the ISF.

6 Summary and Perspectives

Since the original formulation of the "brain glutamate efflux hypothesis" (Pardridge 1979), a number of studies have demonstrated that excitatory amino acid transporters are present in brain endothelial cells both in vitro and in vivo, although the subtype expression pattern varies among species. EAAT-mediated L-glutamate uptake into the endothelial cells constitutes the first step of the brain L-glutamate efflux; however, the following steps remain unclear. Saturable, low-affinity luminal uptake has been demonstrated, indicating the presence of an L-glutamate transporter in the luminal membrane of endothelial cells. Such a transporter could also in theory account for L-glutamate transport from endothelial cell interior to blood, given an L-glutamate concentration gradient from cell to blood which would favor this direction of transport. However, it has also recently been demonstrated that some metabolism of L-glutamate occurs in the endothelial cells (and/or the astrocytes), followed by efflux of the metabolism products to the blood. Experimental evidence suggests that the glutamate-glutamine cycle does not play a role in the brain efflux of L-glutamate. However, metabolism to α-ketoglutarate via aminotransferases or glutamate dehydrogenase is likely to take place both in endothelial cells and astrocytes. The relative contributions of transport and metabolism to the apparent L-glutamate efflux have not been fully elucidated and warrant further studies.

A number of in vivo studies have indicated that scavenging of blood glutamate could improve the outcome after stroke, subarachnoid hemorrhage, traumatic brain injury, and paraoxon intoxication. The mechanism suggested to be responsible for this phenomenon is transporter-mediated brain glutamate efflux, i.e., when blood glutamate concentrations are kept low, the concentration gradient for transporter-mediated brain-to-blood efflux is kept favorable for efflux, but as yet no firm in vivo evidence exists to support this point of view. A direction for future studies would be to focus on verifying the presence of EAATs on brain endothelium in vivo, determine EAAT subtype expression profile, identify the possible transporter responsible for the transport of L-glutamate across the luminal membrane, and investigate to what extent metabolism and transporter-mediated transport of L-glutamate contributes to the apparent L-glutamate efflux. This could lead to a clarification of the mechanisms behind the apparently beneficial effects of blood L-glutamate scavenging.

Conflict of Interest The author declares no conflicts of interest.

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