REVIEW

Alterations of Blood Brain Barrier Function in Hyperammonemia: An Overview

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Abstract Ammonia is a neurotoxin involved in the pathogenesis of neurological conditions associated with hyperammonemia, including hepatic encephalopathy, a condition associated with acute—(ALF) or chronic liver failure. This article reviews evidence that apart from directly affecting the metabolism and function of the central nervous system cells, ammonia influences the passage of different molecules across the blood brain barrier (BBB). A brief description is provided of the tight junctions, which couple adjacent cerebral capillary endothelial cells to each other to form the barrier. Ammonia modulates the transcellular passage of low-to medium-size molecules, by affecting their carriers located at the BBB. Ammonia induces interrelated aberrations of the transport of the large neutral amino acids and aromatic amino acids (AAA), whose influx is augmented by exchange with glutamine produced in the course of ammonia detoxification, and maybe also modulated by the extracellularly acting gamma-glutamyl moiety transferring enzyme, gamma-glutamyl-transpeptidase. Impaired AAA transport affects neurotransmission by altering intracerebral synthesis of catecholamines (serotonin and dopamine), and producing "false neurotransmitters" (octopamine and phenylethylamine). Ammonia also modulates BBB transport of the cationic amino acids: the nitric oxide precursor, arginine, and ornithine, which is an ammonia trap, and affects the transport of energy metabolites glucose and creatine. Moreover, ammonia acting either directly or in synergy with liver injury-derived inflammatory cytokines also evokes subtle increases of the transcellular passage of molecules of different size (BBB "leakage"), which appears to be responsible for the vasogenic component of cerebral edema associated with ALF.

Keywords Ammonia neurotoxicity · Hepatic encephalopathy · Blood–brain barrier · Amino acids · Vasogenic brain edema

Introductory Comments

Homeostasis of the brain is maintained owing to its rigidly controlled communication with the peripheral tissues. Entry of metabolites from the periphery to the brain is controlled by the blood brain barrier (BBB). The major structural constituents of the BBB are the cerebral microvascular endothelial cells, and their barrier function relies on so- called "tight-junctions" (TJs), consisting of transmembrane components: junctional adhesion molecule (JAM)-1, occludin, and the claudins and intracellular proteins: ZO-1, ZO-2, and ZO-3, which link transmembrane proteins to the actin filaments of cytoskeleton and in this way improve stability and functioning of the TJ. Adherent junctions which are located in the basal region below the TJs, also contribute to the barrier function. Cadherins stabilize adhesion between neighboring endothelial cells, while intracellularly, catenins link cadherins to the cytoskeleton (Fig. 1). The BBB is both physical and metabolic in its nature. Physically, the TJs limit free paracellular diffusion of low molecular weight compounds and make the transcellular transport of larger molecules dependent on specific transport systems, which can be grouped

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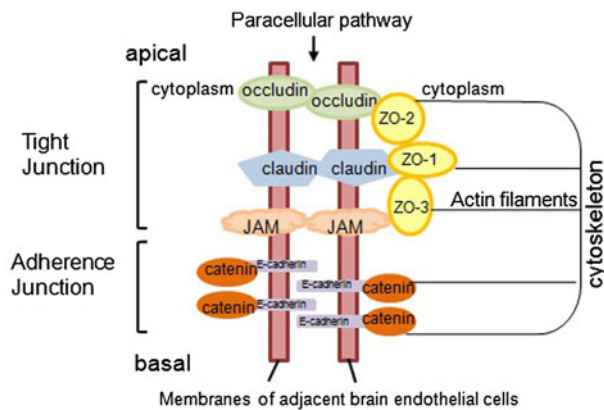e-mail: jalb@cmdik.pan.pl

Fig. 1 Composition of the tight junction and adherence junction which collectively restrict the paracellular passage of solutes across the BBB

accordingly to the class of molecules transported (Hawkins and Davis 2005; Carvey et al. 2009). These transport systems are located in endothelial cells, and are modulated both intrinsically and by other cells of the neurovascular unit: astrocytes and pericytes (Simard and Nedergaard 2004). Fine-tuning of the transport involves its polarization by differential location of the transport systems in the luminal versus abluminal membranes, which holds in particular for the different amino acid transport systems (Hawkins et al. 2006). In this way two ultimate and complementary goals are reached: (i) control of the inflow and outflow of metabolic precursors and products, (ii) prevention of entry to the brain of undesired compounds.

The sections below describes the evolution of views on the role of BBB changes in the pathogenesis of diseases associated with increased exposure of the brain to blood-derived ammonia. "Studies on BBB penetration by different compounds in HE models: a historical account" section gives a historical perspective on the experimental studies on ammonia- and HE-induced changes in BBB penetration of different compounds, without emphasis on the underlying mechanisms. "Transcellular passage of different molecules across the endothelium: roles of active transport" section of the review will elaborate on the relatively well explored subject of modulation of transcellular passage, which represents active transport of medium- to large-molecules, and channel- or transporter-mediated ion fluxes across the capillary endothelial cell membranes. "BBB leakage induced by ammonia and inflammatory molecules: new vistas on the underlying mechanisms" section is devoted to the new findings regarding the mechanisms underlying alterations in the paracellular transport which is defined as BBB leakage, the role of which in ammonia neurotoxicity has so far been underestimated.

Studies on BBB Penetration by Different Compounds in HE Models: A Historical Account

Pioneering studies pertinent to the effect of ammonia on BBB permeability were performed on animals with porta-caval anastomosis (PCA)—a model which mimics the condition of portal-systemic shunting in patients with liver cirrhosis. Laursen et al. (1975) showed that BBB in PCA rats is leaky to horseradish peroxidase (HRP). This observation has been confirmed by Sumner (1982) in a similar experimental setting, and by others using different BBB permeability markers and/or HE models: by Zaki (1983) also in PCA rats who measured amino acid influx using the Oldendorf perfusion technique (Oldendorf 1971), and by Horowitz et al. (1983) in galactosamine-induced animal model of acute liver failure (ALF), where permeability changes to α—aminoisobutyric acid were measured. However, other contemporary animal studies often performed in similar HE models and using similar markers, revealed no brain vascular permeability changes. Examples include the absence of changes of sucrose and methyl-aminoisobutyric acid permeation in galactosamine—induced HE (Lo et al. 1987), and to mannitol or ions in the PCA model (Sarna et al. 1977; Alexander et al. 2000). As will be discussed in "Transcellular passage of different molecules across the endothelium: roles of active transport" and "BBB leakage induced by ammonia and inflammatory molecules: new vistas on the underlying mechanisms" sections, controversies about the BBB status as assessed with different compounds have lasted until the present time, with BBB changes being either confirmed (Wang et al. 2011) or denied (Goldbecker et al. 2010).

Incoherent results were also obtained with regard to the passage of ammonia through the BBB, as monitored with ^{13}N-labeled ammonia (PET technique). Lockwood et al. (1991) showed that ammonia enters the brain more easily in advanced HE patients than in healthy controls. By contrast Goldbecker et al. (2010) did not see any differences in BBB permeability for ammonia between patients with and without liver failure. Sørensen et al. (2007) observed increased ammonia accumulation in cirrhotic patients, but in their hands the increase was solely attributable to increased blood ammonia content.

Understanding of the effects of hyperammonemia on ammonia passage will require separate analysis of the two different forms of ammonia. At physiological pH overwhelming proportion of ammonia occurs as a cation (Warren 1962), so it enters the brain mainly by a transcellular route, using an array of potassium channels and transporters or by substituting other cations with similar hydrated radius (Ott and Larsen 2004). One article indicated the presence of a specific NH_4^+ carrier the Rhesus associated glycoprotein RhCG in the brain capillaries

(Huang and Liu 2001) but its location (luminal vs. abluminal side) and functionality remains to be confirmed. However, recently the pericellular penetration by gaseous ammonia is being taken under consideration as a significant alternative (Ott and Larsen 2004). It is not known which of the two routes would be affected under excessive ammonia load.

On the top of these controversies, increased vesicular transport across endothelial cells and swelling of astrocytic end-feet has been observed in different HE models (Pilbeam et al. 1983), with TJs remaining intact (Kato et al. 1992). It would thus appear that altered transcellular passage maybe a frequent phenomenon, albeit BBB changes in HE are often too subtle to be detected with markers of gross BBB leakage.

Transcellular Passage of Different Molecules Across the Endothelium: Roles of Active Transport

Amino Acids

PCA in rats is associated with amino acid imbalance in CSF and brain due to enhanced blood to brain transport of tryptophan and other members of large neutral amino acid group (LNAA) (James et al. 1978). In addition, increased concentration of aromatic amino acids (AAA) was found in brains of rats with PCA, while the level of branched-chain amino acids was decreased (Smith et al. 1978). The above observations prompted a hypothesis that these alterations may contribute to impaired neurotransmission in HE by producing (i) excessive amounts of neurotransmitters from which they derive, and/or (ii) "false" instead of "authentic" neurotransmitters, which are similar in structure but are either not active at the postsynaptic membrane, or their activity differs from their "true" counterparts (Curzon et al. 1975). The "false neurotransmitter" hypothesis appears attractive, because AAA are also precursors of these "false" modulators: tyrosine for octopamine; phenylalanine for phenylethanolamine. Indeed, James et al. (1976) and Cangiano et al. (1982) showed elevated brain octopamine and phenylethanolamine levels in the brains of PCA rats, and Hilgier et al. (1985) in the brains of rats with thioacetamide-induced liver failure. However, the contribution of these false neurotransmitters to the neurotransmission imbalance associated with HE has insofar not been examined in more detail.

A plausible explanation for the ammonia-induced increase of blood–brain AAA transport activity was proposed by James and colleagues (1979) who hypothesized that during hyperammonemia, increased brain glutamine (Gln) production is followed by increased Gln efflux from the brain, resulting in increased inward transport of these amino acids. This inference has been proven directly in studies in which increased tryptophan (Try) uptake in exchange with Gln via the L-transport system was recorded in cerebral capillary microvessels isolated from PCA rats (Cangiano et al. 1983). Vice versa, release of newly loaded Gln from the capillaries was promoted by the Try and leucine (Leu), and the effect was more pronounced when the capillaries were isolated from TAA rats or following their incubation with ammonia than in control preparations (Hilgier et al. 1992). In the in situ setting, Rigotti et al. (1985) showed that treatment of PCA rats with an inhibitor of Gln synthesis, methionine sulfoximine (MSO), reduced the increased accumulation of the AAA in the brain in a manner correlated with increased ammonia accumulation. Furthermore, Hawkins et al. (1993) showed that administration of MSO to PCA rats normalized amino acid imbalance ascribed to excessive Gln production.

Hyperammonemia was shown to be directly responsible for PCA-induced alterations in the metabolism and transport of amino acids (Jessy et al. 1990), including elevated brain Try content and rise in the brain level of a serotonin metabolite, 5-hydroxyindoleacetic acid. These effects appeared to be due to ammonia-induced functional impairment of LNAA transport at the BBB. In rats in which HA was executed by urease administration, the impairment was found closely correlated with the rise in brain Gln content (Bachmann and Colombo 1983), while in the cortical capillaries the increased Try-Gln exchange could be related to the raised γ-glutamyl-transpeptidase (GGT) activity (Stastný et al. 1988). Because GGT participates in LNAA transport and its activity was found to be increased in brain capillaries from hyperammonemic rats, a hypothesis has been put forward that GGT is involved in triggering the outward transport of the excess of Gln from brain (Gorgievski-Hrisoho et al. 1986). In this way, enhanced activation of GGT could contribute to raised Try and other LNAA levels as observed in rats with TAA-induced ALF (Hilgier et al. 1990). The above speculations were confirmed in a follow up study from the author's laboratory showing that GGT affects the L system-mediated amino acid exchange (Hilgier et al. 1992).

The BBB transport of the cationic amino acids arginine (Arg) and ornithine (Orn) was investigated in different HE models, and contradictory results have been obtained. Zaki et al. (1984) showed a 30% increase in the brain uptake of Arg in the galactosamine model of hepatic failure; however, the effect was not specific to this amino acid and possibly secondary to BBB leakage also revealed by high molecular weight markers. By contrast, Arg uptake from blood to brain was found decreased in chronic HE (PCA) rats (Zanchin et al. 1979) and in rats with thioacetamide (TAA)-induced HE (Albrecht et al. 1996). With regard to Orn, increased brain uptake index of this amino acid

coincident with its increased content in the blood was found in the TAA model of HE (Albrecht and Hilgier 1986; Albrecht et al. 1994 1996; Albrecht et al. 1996). Increased BBB transport of Orn in the TAA model has been considered as auto-protective response and in the same line has been speculated to facilitate intracerebral therapeutic action of the ammonia-trapping drug, L-ornithine-L-aspartate (LOLA) (Albrecht et al. 1994; Albrecht et al. 1996). However, the benefits of Orn may not apply to HE in a chronic setting, where blood to brain transport of Orn appears to remain unchanged (Zanchin et al. 1979).

The mechanism underlying alterations of BBB transport of Arg and Orn has been hypothesized to involve changes in the basic amino acid transporter y^+ activity and competition between these two amino acids for the transport site (Albrecht et al. 1996), but experimental evidence in support of this hypothesis has not been provided as yet. The effects of HE on Arg transport are also likely to be mediated by Gln, which accumulates intracerebrally in consequence of increased ammonia influx (Cooper and Plum 1987), overloading different cellular and subcellular compartments of the CNS (Albrecht 2010). It has been shown that Gln added exogenously reduces NO generation in the brain by inhibiting Arg transport via the Arg/Gln exchanger, y^+LAT2, and that this effect is potentiated when ammonia is infused directly to the brain (Hilgier et al. 2009), or accumulates in there during HE (Zielińska et al. 2011). If the above mechanism operates not only in the CNS cells but also in the cerebral capillary endothelial cells forming the BBB, enhanced Gln accumulation would modulate Arg transport in these cells. The final outcome of this interaction would depend on whether Gln accumulates intra- or extra-cellularly. A hypothesis that such an interaction may occur is supported by the observation that, Gln infusion in the absence of hyperammonemia impairs cerebrovascular CO_2 reactivity, most likely by reducing Arg availability and NO synthesis, because co-infusion of Arg counteracts the effect caused by glutamine (Okada et al. 2000). Consistent with the role of Arg/Gln exchange at the BBB, our preliminary data indicate that ammonia increases the expression of the y^+LAT2 transporter in a cerebral capillary endothelial cell line (manuscript in preparation), as it does in the brain in the course of HA in situ (Zielińska et al. 2011).

Further studies on the mechanisms and pathophysiological implications of the changes in Arg or Orn influx to the brain are warranted in view of the proven or suspected contributions of the amino acids to the pathogenesis of HE. Arg is a precursor of NO, a compound whose increased accumulation is engaged in the inflammatory response of the brain to ammonia (Jalan et al. 2011), and in ammonia-induced brain swelling (Häussinger and Görg 2010), while decreased NO synthesis has been implicated in impairment

of cognition associated with prolonged hyperammonemia (Felipo 2006). Moreover, HA increases Arg uptake to the different cell types within the CNS (Rao et al. 1997; Hazell et al. 1998), and HE in the TAA model stimulates Arg conversion to the neurotransmitter amino acids Glu and GABA as measured in the whole brain (Albrecht and Hilgier 1986) and in synaptosomes derived from these rats, which is likely to alter the balance between the inhibitory and excitatory neurotransmission (Albrecht et al. 1990). Evaluation of the contribution of changes in Arg transport across the BBB to the availability of this amino acid in the brain cannot be accomplished without accounting for the variability in blood Arg content in the different hyperammonemic models. The plasma Arg level was shown to be decreased in PCA rats (Zanchin et al. 1979), but was elevated in rats subjected to prolonged hyperammonemia (Ishihara et al. 1998), and fluctuated from increase to decrease during the development of TAA-induced HE (Albrecht and Hilgier 1986).

Orn plays a role in ammonia detoxification and gives rise to polyamines which exert hepato- and neuroprotection (Sikorska et al. 2010; Seiler 2000). Treatment with LOLA, where Orn contributes to urea formation, reduces blood ammonia level and in consequence improves the general condition of HE patients (Kircheis et al. 2002; Toris et al. 2011). In this light, increased brain uptake of Orn as found in the TAA model of HE would further promote protection (Albrecht et al. 1994).

Orn also contributes in some degree to the biosynthesis of the neurotransmitter amino acids Glu and GABA (Shank and Campbell 1983). Similar to Arg, conversion of its product Orn to Glu/GABA is stimulated during HE (Albrecht and Hilgier 1986; Albrecht et al. 1990). However, implications for this increased conversion for neurotransmission imbalance associated with HE are not known.

Taurine (Tau) is a sulfur amino acid largely implicated in osmoregulatory and neuroprotective responses of the brain in various diseases, including hyperammonemia and HE (Bosman et al. 1992; Butterworth 1996; Faff et al. 1997; Zielinska et al. 2003). Volume regulatory properties of Tau are thought to be of particular importance in the case of brain edema, a major consequence of hyperammonemia, which results from impaired water homeostasis followed by swelling of astrocytes (Blei 2005). HE but not HA was associated with elevated blood content and increased brain uptake from blood to brain of Tau, which collectively contributed to the increase of Tau level in cerebral cortex (Hilgier et al. 1996). Similar observation that liver failure induces elevation of Tau in the blood were also made by other authors (Hamberger and Nyström 1984; Zimmermann et al. 1989). Because increased passage of Tau was not due to massive breakdown of BBB

(as manifested by the absence of penetration of L-aspartate, which is not transported by intact capillary endothelial cells), it was believed to reflect activation of a Tau transport system (Hilgier et al. 1996). Of note in this context, treatment of an endothelial cell line with ammonia led to up-regulation and increased function of Tau transporter (Bélanger et al. 2007).

Energy Substrates

Hyperammonemia by affecting BBB transport of different substances and molecules can also lead to disturbances in cerebral energy homeostasis. Hepatic encephalopathy evoked by PCA was demonstrated to be associated with decreased brain glucose use and energy metabolism (DeJoseph and Hawkins 1991), and a similar effect was noted in rats with TAA-induced HE (Hilgier et al. 1991). Brain uptake index of glucose was reduced after PCA in rats (Sarna et al. 1979; Crinquette et al. 1982) and this decrease was almost entirely due to the decrease in plasma glucose concentrations (Mans et al. 1986). GLUT-1, the principal glucose transporter at the BBB responsible for supplying CNS cells with blood-borne glucose was demonstrated to be induced by ALF (Bélanger et al. 2006). Since inhibition of glucose oxidative metabolism and subsequent activation of cerebral glycolysis are a hallmark of brain energy metabolism in HE animals (Zwingmann et al. 2003; Rao and Norenberg 2001), increased expression of GLUT-1 maybe considered as a compensatory response aimed at supporting higher glycolysis and maintaining brain ATP levels.

Creatine (Cr) a key substrate of the creatine/phospho-creatine/creatine kinase pathway is involved in regeneration of ATP and in this way it also contributes to brain energy metabolism. Moreover, Cr was shown to affect GABA-ergic neurotransmission by acting as partial agonist on post-synaptic GABA(A) receptors (Cupello et al. 2008) and to be crucial in dendritic and axonal elongation (Braissant et al. 2002). Exposure to ammonia was shown to generate a deficiency in Cr in CNS cells and to lead to neuronal cell loss, while co-treatment with Cr was neuroprotective under ammonia exposure, but only in the presence of astrocytes (Braissant 2002). Ammonia treatment was demonstrated to increase Cr uptake in cultured microcapillary brain endothelial cells (Bélanger et al. 2007), which probably reflects a neuroprotective response.

BBB Leakage Induced by Ammonia and Inflammatory Molecules: New Vistas on the Underlying Mechanisms

Recent studies confirmed the view that hyperammonemia produces subtle changes in BBB integrity and partly unraveled the underlying mechanism. Brain extravasation and edema in azoxymethane-induced ALF were found to be secondary to tight junction (TJ) protein degradation mediated by activation of matrix metalloproteinase-9 (MMP-9) (Nguyen et al. 2006). Specifically, it has been shown that TJ proteins occludin and claudin-5 are significantly degraded in the brains of mice with galactosamine-induced ALF, and this effect was reversed by treatment with inhibitor of MMP-9, GM6001 (Chen et al. 2009). A recent study delineated the most likely sequence of events linking activation of MMP-9 to occludin degradation in ALF mice; the intermediate steps include transactivation of epidermal growth factor receptor (EGFR) and p38 MAPK/NFκB (mitogen-activated protein kinase/nuclear factor-kappa B) (Chen et al. 2011). Cauli et al. (2011) observed that progression of intracranial pressure in the course of ALF is strictly correlated with the increase in BBB permeability and MMP-9 content. Basing on this study the authors proposed a sequence of events of ALF-induced brain damage, in which increase in BBB permeability is an initial step leading to vasogenic edema followed by ammonia excitotoxicity and cytotoxic edema.

Inflammatory molecules, including cytokines (IL-1 and/or IL-6) and tumor necrosis factor-alpha (TNF-α) are increased in plasma during acute and chronic liver failure in patients (Tilg et al. 1992; Wright et al. 2007), and in animals with experimentally-induced HE (Jiang et al. 2009). Circulating levels of TNF-α correlate positively with the severity of HE (Odeh et al. 2005), moreover, its involvement in the development of intracranial pressure in patients with ALF was demonstrated (Jalan et al. 2004). Plasma IL-6 level was also found well correlated with the severity of HE and morbidity of the patients (Sheron et al. 1991). Because massive breakdown of BBB is not observed during HE, it is believed that the effects of inflammatory cytokines are transduced to the CNS by vasoactive agents such as nitric oxide or prostanoids, which are synthesized by BBB-forming endothelium (Licinio and Wong 1997). However, TNF-α was recently shown to directly affect the blood–brain barrier permeability in ALF animals in the galactosamine (Lv 2010) and APAP model (Wang et al. 2011) and in human ALF patients (Lv 2010), by disrupting TJs and inducing loss of the TJ-associated protein occludin (Lv 2010).

Conclusions and Perspectives

Data presented in this review provide considerable evidence that ammonia alters the passage of different molecules across the BBB, both by the transcellular route representing active or facilitated transport, and paracellularly, which occurs due to changes in the integrity of BBB constituents and thus reflects BBB leakage. As discussed above, increased BBB permeability adds a vasogenic

component to the cytotoxic brain edema associated with HE (Cauli et al. 2011).

The effects of ammonia on the carrier-mediated transport of different molecules by the cerebral endothelial cells have been studied in considerable detail and the outlines of the changes in amino acid or energy metabolite transport are relatively well described. By contrast, the transcellular transport has long been given little consideration, mainly because in most HE models, the ammonia-or HE-induced changes have been too subtle and spatially restricted to be visualized by standard light- and electron microscopic techniques. The advent of more sensitive techniques has made it possible to identify the changes in TJ proteins and their environment in a microscale, and provided tools to bridge the observations to the molecular mechanisms underlying the BBB leakage. Further studies in this direction should allow to distinguish between the BBB changes in HE which are induced directly by ammonia and those related to inflammatory toxins, mostly cytokines. One aspect deserving consideration in the future studies is the potential role of free radicals of oxygen and nitrogen, which have been found to be generated in excess by ammonia in different models and cell types of the CNS and are responsible for the oxidative/nitrosative stress (ONS) (Bemeur et al. 2010; Häussinger 2010; Skowrońska et al. 2010). Preliminary results from our laboratory disclosed that ONS markers accumulate in an ammonia-treated brain microvascular endothelial cell line and increase permeability of these cells to a high molecular weight marker (Skowrońska et al., manuscript in preparation). This line of investigation appears attractive in view of the fact that ONS causes BBB dysfunction in brain pathologies of varying etiology and severity (Lehner et al. 2011). Many of the intracellular derangements known to be induced by ammonia in the cells within the CNS or in peripheral tissues are likely to hold for the BBB-forming cerebral vascular endothelial cells, and may converge with events triggered in the different cells by ONS. Of note activation of the p38 MAPK/NFκB pathway which underlies MMP-9-induced TJ protein damage (Chen et al. 2011), is also involved in ammonia-induced oxidative damage of astrocytes (Jayakumar et al. 2006; Sinke et al. 2008). Other targets may include, for instance, altered Nrf2-mediated synthesis of heme oxygenase I, an effect common to the response to various blood brain barrier damaging conditions (Lehner et al. 2011) and to the ammonia-induced ONS in astrocytes (Warskulat et al. 2002). Clearly, the above described mechanisms do not exhaust the list of possibilities that are worth further investigation.

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