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Iron transport across the blood-brain barrier; Development, neurovascular regulation and cerebral amyloid angiopathy

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Abstract

There are two barriers for iron entry into the brain: 1) the brain-cerebrospinal fluid (CSF) barrier and 2) the blood-brain barrier (BBB). Here, we review the literature on developmental iron accumulation by the brain, focusing on the transport of iron through the brain microvascular endothelial cells (BMVEC) of the BBB. We review the iron trafficking proteins which may be involved in the iron flux across BMVEC and discuss the plausible mechanisms of BMVEC iron uptake and efflux. We suggest a model for how BMVEC iron uptake and efflux are regulated and a mechanism by which the majority of iron is trafficked across the developing BBB under the direct guidance of neighboring astrocytes. Thus, we place brain iron uptake in the context of the neurovascular unit of the adult brain. Last, we propose that BMVEC iron is involved in the aggregation of amyloid- β peptides leading to the progression of cerebral amyloid angiopathy which often occurs prior to dementia and the onset of Alzheimer's disease.

Keywords

Iron; Blood-brain barrier; Astrocytes; Transferrin; Amyloid precursor protein

Introduction

The innate ability of iron to act as both an electron acceptor and electron donor makes it essential to life. The requirement for iron extends to all cell types of the brain. Oligodendrocytes for example, exhibit an intrinsic thirst for iron as indicated by their high levels of the iron storage protein ferritin [1]; oligodendrocyte iron participates as a co-factor in the myelination of axons throughout development [2]. Given the participation of iron as a cofactor in myelination, mitochondrial energy generation, neurotransmission, oxygen transport, and cellular division, a disruption in normal iron trafficking is detrimental to normal brain function [3, 4]. In addition, iron is likely a factor in several neurological disorders such as Parkinson's disease, Alzheimer's disease, and aceruloplasminemia [4-7]. Expanding our knowledge of the mechanisms involved in brain iron trafficking is a necessary step towards understanding iron-related neurological diseases.

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There are two barriers for iron entry into the brain: 1) the brain-cerebrospinal fluid (CSF) barrier and 2) the blood-brain barrier (BBB). The BBB is composed of different cell types acting in unison to form a tight barrier which, under normal conditions, is impenetrable to polar molecules of the blood. The cell types which constitute the BBB include brain microvascular endothelial cells (BMVEC), astrocytes, and pericytes [8, 9]. In the periphery, vascular endothelial cells are often fenestrated and lack tight junctions allowing for the paracellular flux of polar molecules into the surrounding tissue [8]. BMVEC lack fenestrations and possess tight junctions, forcing most molecules to be trafficked transcellularly *via* receptor-mediated transcytosis (insulin, ferritin), adsorptive transcytosis (albumin), or transport proteins (glucose, amino acids) [8-11].

The morphology inherent to BMVEC allows for the formation of two distinct surfaces; the apical membrane (blood-side) and the basal membrane (brain-side). Located basolateral to BMVEC are astrocytes. These glial cells are thought to act as buffers in the brain, protecting neurons from harmful chemicals, ROS, etc. [12, 13]. Recently, our lab has demonstrated that astrocytes, which eventually encapsulate the BMVEC [10], also regulate the basolateral efflux of iron from a human brain microvasculature cell line (hBMVEC) [14]. This hBMVEC iron efflux is regulated by astrocyte-secreted agents that either enhance (ceruloplasmin (Cp)) or suppress (hepcidin) activity [14]. In addition to Cp, proteins endogenous to astrocytes that may also stimulate hBMVEC iron efflux include ferritin and amyloid- β precursor protein (APP) [13, 15]. This review combines recent observations to support our proposed developmental model of astrocyte-modulated iron trafficking by hBMVEC.

In addition, we propose iron trafficking across the BBB in the adult mammal is modulated by the dynamic iron requirements of the neurovascular unit. The neurovascular unit, composed of BMVEC, astrocytes, and neurons, is an integral cluster of cells which are able to communicate *via* juxtacrine signaling. We suggest the neurovascular unit responds directly to the substrate requirements of the cells within that unit. For example, dynamic adaptation to neuronal nutrient iron deprivation may involve direct signaling from neurons to astrocytes to BMVEC, increasing the rate of brain iron uptake across the BBB. Here, we discuss the mechanisms of brain iron accumulation *via* the BBB in response to both astrocyte proximity and metabolic changes within the neurovascular unit. We conclude by outlining a model for how iron may exacerbate amyloid- β (A β) aggregation in the vicinity of BMVEC.

Proteins involved in BMVEC iron uptake

Due to the stringent tight-junction properties of the BBB, there must exist a cell-based mechanism for trafficking iron across this barrier. Overall, this mechanism involves two transmembrane steps: iron uptake into the BMVEC at the apical (blood) surface followed by iron efflux into the brain interstitium at the basolateral (brain) surface. There are two possible mechanisms for iron uptake into BMVEC. The first is referred to as transferrin-bound iron (TBI) uptake involving transferrin (Tf) endocytosis. The second is uptake of iron from non-transferrin bound iron (NTBI), a process that involves an iron transporter at the apical membrane. TBI iron can be released within the cell via canonical endosomal

acidification, ferric iron reduction and efflux into the cytoplasm to enter the pool of iron that also includes NTBI iron accumulated by uptake at the plasma membrane. Another possible process is Tf transcytosis in which iron remains bound and the holo-Tf is released at the basal surface by exocytosis; this pathway would supply holo-Tf to the brain interstitium, that is, Tf uptake would parallel iron uptake. Iron released into the cytoplasm, whether by direct uptake at the apical membrane, or release from endosomes, would become substrate for an iron efflux protein (Fig. 1). We will review first the proteins linked to pathways of iron uptake throughout the central nervous system (CNS) (Table 1).

Transferrin and transferrin receptor

Transferrin is an 80 kDa bilobal iron binding glycoprotein. A single ferric iron atom can reversibly bind either lobe of Tf (C- or N-lobe) with high affinity ($\sim 10^{22} \text{ M}^{-1}$ at pH 7.4) [16]. Release of iron from Tf can be potentiated through decreased pH, exogenous ligands, and the binding of Tf to its receptor [17-19] (see “*Transferrin and transferrin receptor recycling-dependent BMVEC iron uptake pathways*” section for details). In the brain, both Tf transcript and protein have been identified (Table 1). Abundant Tf mRNA exists in oligodendrocytes, neurons, and astrocytes. Synthesis and secretion of the protein however, only occurs with oligodendrocytes and astrocytes [20-22]. Tf has been identified in BMVEC [23]; likely a result of receptor-mediated endocytosis of serum Tf. The majority of serum iron is bound and transported by Tf to cells expressing TfR. Expression of TfR is post-transcriptionally regulated. The TfR transcript contains 5 iron-responsive element (IRE) stem-loops in its 3' untranslated region, which together form an IRE stability element [24-26]. Binding of the iron responsive protein (IRP) to the TfR IRE under low cytosolic iron conditions decreases the nucleolytic turnover of the transcript allowing for increased protein production and Tf-iron uptake [27].

There are approximately 100,000 TfR per primary bovine BMVEC [28]. Expression of TfR by BMVEC adapts to changes in age and iron status [29-34]. TfR have been identified in rat BMVEC at postnatal days 5, 10, and 15 [34]; in contrast, BMVEC of 8 week old rats are depleted of TfR [33]. ^{59}Fe entry into the brains of rats intravenously injected with ^{59}Fe - ^{125}I -Tf reached a maximum at day 15 and significantly decreased thereafter with minimal accumulation observed in brains of 8 week old rats [35], reflecting the developmental changes in BMVEC TfR expression. Iron provided to hBMVEC *in vitro* as ^{59}Fe -Tf is accumulated in nearly five-fold excess to ^{59}Fe -citrate [36]. These data suggest that Tf-bound iron is the primary substrate for brain iron accumulation through postnatal ontogenesis when TfR expression in BMVEC is highest, and less so in adulthood when BMVEC TfR expression is low.

Metalloreductases and ferri-reduction

First identified in erythroid cells in 2005, the six transmembrane epithelial antigen of the prostate 3 (Steap3) was shown to co-localize with Tf, TfR, and epitope-tagged divalent metal transporter 1 (DMT1) [37]. Reduction of Tf-iron requires the donation of an electron from an endogenous reductant; the Steap family of ferrireductases acts as courier for this electron. Each member of the Steap family of metalloreductases (Steap 2-4) except for Steap1 contain a unique flavin-NAD(P)H binding domain located near their cytosolic N-

terminus; this domain shares homology to archaeal and bacterial $F_{420}H_2:NADP^+$ oxidoreductase binding domains [38]. Endogenous reductants will bind to the cytosolic flavin-NAD(P)H binding domain and initiate the donation of an electron to the metalloredutase. Conserved throughout each of the Steap family members is a single heme group within the transmembrane region of the protein [37]. The heme group is putatively bound by two heme-binding histidine residues. Upon mutation of each histidine residue to leucine, electron transfer is lost as indicated by reduced ferrireductase activity [37]. These data suggest that the electron from the endogenous reductant is shuttled from the flavin-NAD(P)H binding domain, through the heme of the protein, and subsequently to the endosomal iron.

Using quantitative real-time PCR on human cDNA samples, the relative mRNA abundance of all of the STEAP family of metalloredutases has been mapped [38]. Each Steap protein was represented by a relatively high abundance of mRNA in the human prostate and/or fetal liver, however only STEAP2 mRNA was abundantly detected in the brain [37-39]. Indirect immunofluorescence has identified Steap2 on the plasma membrane of hBMVEC [40]. Epitope-tagged Steap2 transfected into HEK293T cells yielded a strong co-localization with both Tf and TfR in the endosome [38] suggesting that Steap2 may participate in the reduction of Tf iron in the endosome.

Prior to the characterization of the Steap family of ferrireductases [38, 41], the only characterized mammalian ferrireductase resided in the duodenum. This duodenal reductase is an ascorbate-dependent b-type cytochrome ferrireductase (Dcytb) (also known as Cybrd1) [42, 43]. Dcytb catalyzes the reduction of dietary iron for absorption through DMT1 at the luminal surface of intestinal epithelial cells [42, 44]; there is evidence that Dcytb may act to reduce Cu^{II} as well [43]. Uptake of $^{59}Fe^{III}$ by cells in culture is stimulated upon transfection with Dcytb. This effect is blocked by the Fe^{II} chelator ferrozine suggesting Dcytb acts to reduce Fe^{III} to Fe^{II} prior to uptake [44]. Recently, Dcytb expression has been demonstrated in the airway epithelial cells and hBMVEC [40, 45]. The transcripts that encodes Dcytb has been identified in cultured astrocytes from rats and mice [46, 47]. In 2013, Dcytb protein was demonstrated in hippocampal astrocyte endfeet surrounding the BMVEC of rats, potentially providing a source of ferrireduction for BMVEC-released Fe^{III} [48].

In addition to the reduction of extracellular iron by metalloredutases, NTBI has the potential to become reduced by cell-secreted ascorbic acid which will act directly as a ferrireductant. This mechanism has been demonstrated in K562 cells [49] and in primary cultures of rat astrocytes [50]. In the latter study, the data indicated that with Fe^{3+} as iron source, ~50% of the iron accumulated was *via* DMT1 following ferrireduction by ascorbate secreted by the cells. Whether serum ascorbate, or ascorbate secreted into the luminal space by BMVEC, plays a role in iron accumulation by these cells remains to be investigated, however.

Divalent cation transporters

Following reduction of Fe^{III} to Fe^{II} by extracellular reductants or by a cell-surface ferrireductase, Fe^{II} is absorbed through a divalent cation transporter such as DMT1. DMT1 resides in the brush border of the duodenum where it actively transports nutrient iron into

the duodenal epithelial cells [51-54]. DMT1 was first cloned and characterized by the Hediger group in 1997 using the duodenal mRNA of rats maintained on a low iron diet [55]. Using two-microelectrode voltage clamp assays, the Hediger group evoked currents from *Xenopus* oocytes expressing DMT1 using a broad range of substrates; substrates included Fe^{II} , Zn^{II} , Mn^{II} , Co^{II} , Cd^{II} , Ni^{II} , and Pb^{II} [55] (*see* Table 2 for kinetics associated with Fe^{II} , Mn^{II} , and Zn^{II} transport). Transport of these substrates by DMT1 has been substantiated and expanded to include copper [56, 57], although whether DMT1 transports Cu^{I} or Cu^{II} remains a mystery.

Transport of divalent cations through DMT1 is pH-sensitive. The optimum pH for metal transport by DMT1 ranges from pH 5.5 [55, 58] to pH 6.75 [59]. Conditions optimal for iron transport through DMT1 are found in the brush border of the duodenum (pH 6.0 [60] – 6.74 [61]) and inside the cycling endosome (pH 5.6 [17]). There is, however, evidence that metal transport through DMT1 can occur at pH 7.4 [58, 59, 62, 63]; in the absence of a proton gradient it is unclear how DMT1 transports divalent cations.

DMT1 is co-localized with TfR in rat BMVEC [29]. Expression of DMT1 by rat BMVEC has been corroborated using immunohistochemical probes on sections of rat brains from different developmental time points [34]; change in the relative expression of DMT1 by BMVEC throughout development was not noted. While several lines of evidence support the expression of DMT1 by BMVEC [29, 34, 40], such expression remains controversial [31, 64].

The role of DMT1 *in vivo* may best be examined using the Belgrade rat. The Belgrade rat contains a glycine-to-arginine point mutation at residue 185 (G185R) resulting from a missense mutation of the *Nramp2* gene [65]. Cells and tissues of Belgrade rats phenotypically have diminished iron acquisition capabilities [66]. Furthermore, Belgrade rats present with a significant decrease in brain ^{59}Fe uptake as compared to wild-type controls when administered a dose of ^{59}Fe - ^{125}I -Tf into the lateral tail vein [66]. Belgrade rat pyramidal neurons, cortical neurons, oligodendrocytes, and myelin, all contain less iron than wild-type rats [67]. Astrocytes, and oligodendrocytes neighboring BMVEC appear to accumulate iron in the Belgrade rat [67] suggesting these cell types possess compensatory or alternative iron permeases.

A likely candidate credited with this activity is the transmembrane Zrt- and Irt-like protein 14 (Zip14; SLC39A14). While Zip14 was initially characterized as a Zn^{II} transporter [68], Fe^{II} permease activity has been ascribed to Zip14 as well [69, 70] (*see* Table 2 for kinetics). Iron permeation through Zip14 is optimal at pH ~ 7.4 [70, 71]. Zip14 mRNA has been identified in the brains of mice, yet the cellular distribution of the translated protein remains unknown [72]. At the sub-cellular level within HepG2 cells, Zip14 has been localized to the same early and late cycling endosomes as TfR1 [71]. Furthermore, siRNA knockdown of Zip14 in HepG2 cells significantly diminished the uptake of iron from Tf, suggesting that Zip14 has the potential to export the iron from Tf out of the endosome.

Another member of the family of Zrt- and Irt-like proteins, Zip8, is also capable of transporting Fe^{II} [73] (*see* Table 2 for kinetics). Like Zip14, Zip8 mRNA is abundantly

expressed in brain tissues [72]. At the subcellular level, Zip8 is localized to the plasma membrane and intracellular vesicles of transiently transfected HEK293T cells [73]. The optimal pH of iron permeation through Zip8 (pH 7.4) is on par with the pH of blood (pH 7.35 – 7.45) [73]. These data suggest that Zip8 and Zip14 are in optimal positions to sequester iron from the extracellular fluid for use by the cell. While the data suggest that Zip14 exports iron out of the endosome after removal from Tf, both Zip14 and Zip8 have the potential to transport iron into the cell at the cell surface. To date, neither Zip14 nor Zip8 have been identified in BMVEC.

Transferrin and transferrin receptor recycling-dependent BMVEC iron uptake

Iron in the peripheral circulation is primarily bound to Tf as Fe^{III} , its ferric redox state. The reduction and subsequent release of iron from Tf requires that the electrochemical potential (E°) of the complex, which is intrinsically low (<-500 mV [74]), is allosterically made more positive than the E° of the endogenous reductants [75]. The E° of Tf-bound iron can be made more positive through 1) Tf association with TfR, 2) the binding of an exogenous ligand (i.e. citrate), or 3) a decrease in pH [17, 18, 74, 76]. Such conditions are met in the canonical Tf-TfR cycling pathway. In this pathway, holo-Tf binds to the TfR at the cell surface; two holo-Tf molecules will bind to their receptor, the dimeric TfR, at pH 7.4. This binding event triggers a clathrin-dependent endocytosis of the Tf-TfR complex. Protons are pumped into the resulting endosome *via* the action of an H^+ -ATPase [77], subsequently lowering the endosomal pH to ~ 5.6 [17]. The combination of low pH and TfR binding increases the E° by >200 mV [18]. An electron is transported from a cytosolic reductant, such as NADPH, into the endosomal compartment *via* a ferrireductase, effectively reducing the Fe^{III} within Tf. From here, the endosomal Fe^{II} will traffic from the endosome to the cytosol through DMT1 (Fig. 1) [29, 40].

Alternatively, a mechanism describing the transcytosis of the Tf-TfR complex across BMVEC has been proposed (Fig. 1) [64, 78, 79]. In this model, Tf binds to the TfR at the luminal (apical) surface of the BMVEC. The ensuing endocytosis of the Tf-TfR complex is followed by acidification and release/reduction of the Tf-bound iron as described for canonical cycling. However, in the transcytosis model, the Fe^{II} -containing endosome undergoes exocytosis at the abluminal (basal) surface, releasing Fe^{II} into the brain interstitial fluid. The apo-Tf-TfR complex is then retro-transcytosed to the luminal surface of the BMVEC where, at pH ~ 7.4 , apo-Tf is released into the blood.

This model is controversial. At the pH of the brain interstitial fluid (~ 7.24) apo-Tf would likely detach at the abluminal surface of the BMVEC [80]. However, an insignificant fraction of Tf is transported from the blood into the brain interstitium in comparison to the fraction of iron transported [81, 82], making the transcytosis model unlikely. Supporting this conclusion is an experiment using the mouse TfR antibody, 8D3, which triggers a receptor response (i.e. TfR endocytosis). However, immunohistochemical staining along with 3D modeling clearly indicated that the 8D3 antibody infused into mouse brain failed to reach the cerebral parenchyma, i.e. neither did TfR [83]. Furthermore, recent data indicate that hBMVEC iron derived from TBI is exported from hBMVEC *via* a mechanism involving Fpn and an exocytosomal ferroxidase [36]. Removal of endogenous hBMVEC ferroxidase

activity, an event which would not affect transcytosis, inhibits the efflux of Tf-iron from the cell, making the transcytosis model ever more unlikely [36].

In addition to these questions concerning Tf-transcytosis (whether apo- or holo), transport of bulky Tf molecules through the basal lamina formed by BMVEC is likely inefficient. The basal lamina is a secretion of fibronectin, type-4 collagen, and laminin interposed between endothelial cells and astrocytes [84]. This matrix of cellular protein that is about 150-200 nm thick provides an additional barrier in regards to the access Tf has to the brain's interstitial fluid (ISF). Thus, the current data suggest that holo-Tf is not transcytosed across the BMVEC but that TBI iron is reductively released as Fe^{II} prior to being exported from BMVEC through Fpn. Whether this reductive release involves canonical Tf-TfR cycling or the removal of iron from Tf at the apical plasma membrane remains to be determined.

Mechanisms of recycling-independent BMVEC iron uptake from TBI

While the reduction of Fe^{III} from Tf is commonly thought to occur inside the cycling endosome, a fraction of this reduction appears to occur on the cell-surface of BMVEC. This inference is supported by evidence of nonheme ferrous iron accumulation at the luminal surface of rat BMVEC [85] and by the fact that hBMVEC reduce non Tf-bound and Tf-bound iron *via* a cell-surface ferrireductase [40]. Reduction of iron from Tf on at the plasma membrane has been observed in hepatocytes as well [30]. Recently, our lab demonstrated that the reduction of iron from Tf, as measured by the colorimetric ferrozine assay [86], requires the binding of holo-Tf to the TfR on the cell surface of hBMVEC [40]. In addition to the contributions of TfR, an exogenous ligand will increase the E° of the Tf-bound iron and thus increase the potential for reduction [18, 19]. The presence of the exogenous ligand citrate is physiologically relevant to serum [81, 87-89] and ISF [90]. When applied in a physiologically appropriate ratio to holo-Tf, citrate increased (2-fold) the reduction of Tf-bound iron by hBMVEC [40]. Furthermore, reduction of iron from Tf at the BMVEC cell surface would likely require the contribution of a cell-surface ferrireductase; the contributions of Steap2 and/or alternative ferrireductases have yet to be characterized.

Any cell-surface reduction of iron is likely to occur in the absence of a proton gradient. In these instances either DMT1 is transporting iron in the absence of a proton gradient or an alternative divalent cation transporter supports this ferrous iron uptake. For example, Zip8 or Zip14 would compensate for the lack of DMT1 activity in the absence of a proton gradient in as much as these divalent metal ion transporters have functional optima at physiologic pH (above).

Proteins involved in the efflux of iron from BMVEC

As high concentrations of intracellular iron may be toxic to cells, vertebrates have developed mechanisms to export excess iron out of their cells and into their extracellular space. The endothelial cells of the BBB are a polarized cell type. They sequester iron from the blood at their apical surface and release iron into the brain at their basolateral surface. Next, we will provide an overview of the proteins involved in cellular iron efflux that are currently known to exist in the CNS. Their function, and roles in brain iron trafficking will also be discussed. Proteins linked to iron efflux from cells of the CNS are listed in Table 3.

Ferroportin

Ferroportin (Fpn/IREG1/Slc40a1/MTP1) is the only known mammalian cellular iron exporter. The discrepancy in nomenclature is due to the simultaneous discovery of Fpn by three independent laboratories [91-93]. Donovan *et al.* published the discovery of the *ferroportin1* (*Fpn1*) gene in Zebrafish in early 2000 and characterized the translated product, Fpn, as an iron exporter in *Xenopus* oocytes [91]. This iron permease activity was confirmed later that same year [92, 93]. Ferroportin functions to export Fe^{II} from the cell [91], though transport of Mn, Zn, and Co by Fpn has been demonstrated to a lesser extent [94-96]. The efflux of Fe^{II} from Fpn is catalyzed by the oxidation of the Fe^{II} to Fe^{III} via the action of an exocytosomal ferroxidase [97]. Fpn is a protein dimer that localizes to the plasma membrane of cells [98-100]; other, contradictory reports have concluded that Fpn exists as a monomer [101-103].

The most abundant Fpn transcript contains an IRE in its 5' untranslated region [93]. Fpn is post-transcriptionally regulated via the binding of IRP1 to the *Fpn1* IRE under low iron conditions, effectively repressing the translation initiation of the transcript [104]. A minority fraction of Fpn mRNA lack the 5'-IRE; this form, designated *Fpn1b*, has been detected in the mouse duodenum and in the erythroblasts of both human and mouse [105]. Translation of the *Fpn1b* transcript is insensitive to iron levels but, like *Fpn1*, exhibits post-translational sensitivity to the peptide hormone hepcidin [105, 106].

Hepcidin is secreted by the liver in response to increased hepatic iron and binds to its receptor, Fpn, inducing Fpn ubiquitination and subsequent internalization [107, 108]. The reported hepcidin binding motif on Fpn exists in the extracellular loop between transmembrane domains VII and VIII [109]. Site-directed mutagenesis assays have revealed that upon binding to Fpn, hepcidin induces the phosphorylation of tyrosine residues 302 and 303 as well as the ubiquitination of lysine residue 253 causing Fpn internalization and degradation [109, 110]. The exact mechanism of hepcidin-induced Fpn internalization and degradation remains controversial [108, 111]. Once internalized, however, Fpn and hepcidin are trafficked to the lysosome where both are degraded [112]. Efficacy of the post-translational regulation of Fpn by hepcidin can be reduced by the mammalian ferroxidase ceruloplasmin (Cp); this effect is attributed to the enzyme's ferroxidase activity [113], but may involve protein-protein interactions [47]. Permeation of Fe^{II} through Fpn may prevent Fpn ubiquitination and subsequent turnover [97]. In the brain, hepcidin is expressed by astrocytes that neighbor BMVEC and is released into the cleft formed between these cell types to effectively promote the turnover of BMVEC Fpn and thus diminish the import of iron into the brain [14, 114].

Perhaps the most comprehensive examination of Fpn in mammals involves the basolateral surface of the intestinal duodenum. There, Fpn is colocalized and in functional complex with the mammalian ferroxidase hephaestin (Hp). Together, Fpn and Hp work to export iron from the duodenal epithelium into the blood [115]. Beyond the duodenum, Fpn has been identified in several cell types in the CNS. These include BMVEC [14, 33, 36, 116, 117], neurons [116, 118, 119], oligodendrocytes [116, 118-120], astrocytes [47, 116], and the choroid plexus ependymal cells [116, 118]. Fpn transcript and protein have been localized to

mouse BMVEC ensheathed by astrocytic endfeet [116]. Furthermore, analysis of the rat BMVEC transcriptome revealed the presence of SLC40A1 (Fpn) [121]. Examination of the subcellular localization of Fpn revealed its presence on both the apical and basolateral plasma membranes as well as within the cytosol of hBMVEC [36]. These recent data hopefully put to rest the controversy concerning Fpn expression by BMVEC [29, 64, 118, 119].

Recently, our lab identified Fpn in hBMVEC and demonstrated iron efflux from these cells only when Fpn was localized to the plasma membrane [36]. In the absence of an exocytosomal ferroxidase Fpn is turned over and hBMVEC iron efflux is lost [36]. Both hBMVEC Fpn and iron efflux activity can be recovered *via* the addition of an exogenous ferroxidase to the efflux media [36]. Yang *et al.* identified both the Fpn transcript and protein in rat blood vessels [33]; Fpn expression in BMVEC decreased as development progressed, notably around P14. The depletion of BMVEC Fpn at P14 is modulated by hepcidin secreted into the interstitial space between BMVEC and astrocytes [14]. Whether the majority of this hepcidin is provided by BMVEC or astrocytes has yet to be determined. Under conditions of Fpn depletion, hBMVEC exhibit decreased iron efflux capabilities [14, 36]. BMVEC Fpn and iron efflux capabilities can be restored in the presence of astrocytes *via* the addition of the hepcidin antagonist fursultiamine [14, 122]. Taken together, the recent literature would suggest that Fpn is essential for iron transport across the BBB, a process tightly regulated by neighboring astrocytes.

Multi-copper oxidases – ceruloplasmin, hephaestin and zyklopen

The export of Fe^{II} from Fpn requires the action of an exocytosomal ferroxidase. Mammals express two known multi-copper oxidases, Hp and Cp, both of which possess ferroxidase activity. Biosynthesis of both Hp and Cp involves the incorporation of copper into their structures [123-127]. First crystallized in 1996, Cp was found to contain six copper atoms; three type I Cu located in domains 2, 4 and 6, while one type II Cu and two type III Cu make up the trinuclear cluster at the interface of domains 1 and 6 [127]. In Cp, the type I Cu atoms in domains 4 and 6 are catalytically active and involved in catalyzing the transfer of electrons donated by iron through the protein to the trinuclear cluster where oxygen is reduced to H₂O. Hp, a Cp homologue, also has the ability to oxidize iron through a similar catalytic cycle [124].

Recently, a third multi-copper oxidase, named zyklopen (Zp), has been identified in mammals [128]. Zp shares high sequence and functional (ferroxidase activity) identity with Hp and Cp [128]. Each of the copper-binding sites (type I, II, and III) found in Cp are conserved in Zp [128]. First identified in the placenta [129, 130], Zp was recently demonstrated to be highly expressed in the choroid plexus of mouse brain [128], though the cellular distribution of Zp in the brain is currently undefined. The role, if any, Zp plays in brain iron metabolism has yet to be realized.

Hephaestin, demonstrated as being co-localized and in functional complex with Fpn at the basolateral surface of duodenal epithelial cells [115, 131, 132], has more recently been identified in the brains of mammals [133]. Examination of the rat cortex, hippocampus, striatum, and substantia nigra revealed that both Hp transcript and protein were present from

weeks 1 to 28 of development, although their relative abundance varied with development and dietary iron levels [133]. Hp protein has been identified in astrocytes, oligodendrocytes, microglia, and neurons of the substantia nigra [134]. Immunohistochemical analysis of Hp using brains from Cp null mice have demonstrated expression in the choroid plexus, the ependymal cells lining the lateral ventricle, the endothelial cells of the lateral ventricle, and BMVEC [135]. Expression of Hp by BMVEC has been corroborated and high-resolution imagery has localized Hp to the basolateral surface of hBMVEC [33, 36]. In 2010 the Kim lab demonstrated that Hp was depleted from BMVEC of the developing rat brain by postnatal day 14 [33].

Hp may be involved in iron efflux from cells of the CNS. An example of this has been demonstrated using rats lesioned by 6-hydroxydopamine (6-OHDA). The 6-OHDA treatment is a model for Parkinson's disease, and rats which have undergone this treatment present with iron accumulation in the substantia nigra due to loss of Fpn and Hp transcripts in as little as one day post treatment [134]. Furthermore, oligodendrocyte cultures from neonatal mice with mutations in the *hephaestin* gene (sex-linked anemia mice (*sla*)) have perturbed ^{55}Fe efflux capabilities in comparison to oligodendrocytes from wild-type neonates [120]. Oligodendrocytes of the white matter have compensatory mechanisms for Hp depletion in the *sla* mouse; in the *sla* mouse, Cp expression in the white matter and the spinal cord was significantly upregulated, suggesting that Cp filled this compensatory role [120]. Removal of Hp from hBMVEC *via* depletion of copper bioavailability completely abolished the cells ability to efflux ^{59}Fe [36]. Taken together, the recent data from the literature would suggest that Hp is involved in the efflux of iron from cells of the CNS.

Cp is widely known for its role in serum iron oxidation where it exists as an abundant soluble serum α_2 -glycoprotein; Cp contains >95% of the copper found in the plasma [136]. In the periphery, Cp transcript is found mostly in the liver and lung with subtle expression seen in the spleen, testis, and kidneys of mice [137]. In the CNS, Cp transcript has been identified in the cortex, the cerebellum, the midbrain, the hippocampus, the striatum, the substantia nigra, and a robust amount of transcript exists in the eye [137, 138]; in some instances, Cp transcript abundance in these regions changes as a result of age and iron status [138]. At the cellular level, Cp mRNA can be found in the epithelial cells of the pia-arachnoid, hBMVEC, and the ependymal cells of the choroid plexus [36, 137]. Furthermore, a specific subpopulation of astrocytes, namely those that ensheath the brain microvasculature, express Cp transcript [14, 137, 139], suggesting that Cp may play a critical role at the BBB.

Astrocytes express a form of Cp that is tethered to the plasma membrane *via* a glycosylphosphatidylinositol-anchor (GPI) [140]. This GPI-anchor is the result of an alternative RNA splicing event in the C-terminus of Cp; the C-terminal 5 amino acids of Cp are replaced by a 30 amino acid GPI-anchor signal [141]. An interaction between astrocyte GPI-Cp and Fpn has been demonstrated [47]. Astrocytes cultured from Cp null mice lack the ability to efflux iron [47]. These data suggest that astrocyte GPI-Cp acts to export iron through Fpn, though Cp involvement in cellular iron uptake has also been proposed [142, 143].

Along with GPI-Cp, the CNS also contains a secreted (soluble) form of Cp. The concentration of sCp in the CSF has been measured at 5.8 - 6.8 nM [81, 144]. Quantitative MS-based proteomics have revealed significant enrichment of sCp in the primary mouse astrocyte secretome [15]. Astrocyte-secreted sCp has been identified *via* Western blotting and this protein is ferroxidase active [14]. Astrocytes actively stimulate hBMVEC iron efflux through secretion of sCp into the local microenvironment surrounding hBMVEC [14]. While astrocyte-secreted ferritin may also play a role in hBMVEC iron efflux, immunodepletion experiments suggest that sCp is the major player in hBMVEC iron efflux [14, 15, 145, 146]. These data strongly suggest that astrocyte-secreted sCp is critical for proper brain iron accumulation.

Ferritin

Ferritin is a 24-subunit iron transport and storage protein containing various proportions of two subunit types, heavy (FHC; 21 kDa) and light (FLC; 19 kDa) chains [147-150]. The subunit proportions are controlled at the level of transcription, leading to tissue-dependent variation [150, 151]. Evidence suggests that FHC, which contains a ferroxidase center, oxidizes Fe^{II} for FLC which, in turn, mineralizes and stores Fe^{III} [152, 153]. In the brain, ferritin is found in the choroid plexus [154], astrocytes [155], oligodendrocytes, pyramidal neurons, microglia, and BMVEC [1]. Ferritin is trafficked across BMVEC *via* transcytosis from P1-P7; this method of trafficking is significantly diminished after complete ensheathment of the BMVEC by astrocytic endfeet at P14 [10]. Ferritin molecules trafficked across the BBB may be sequestered by oligodendrocytes which accumulate ferritin during postnatal ontogenesis [1]. Oligodendrocytes, which largely utilize FHC [156], may use the ferritin-iron for the synthesis of myelin [2]. Peak myelination by oligodendrocytes occurs at P15, shortly after ferritin trafficking across the BBB ceases [10, 157]. It is possible that these two events, brain ferritin import and peak myelination, are linked.

Amyloid- β precursor protein (APP)

The amyloid- β precursor protein is expressed by neurons, astrocytes, and BMVEC [13, 145, 158], and has been shown to physically interact with Fpn [159]. In 2010, Duce *et al.* demonstrated a significantly increased retention of iron within cortical neurons from APP^{-/-} mice compared to wild type, suggesting that APP plays a role in modulating iron efflux from the neurons of mice. They described APP as being ferroxidase-active and assigned this activity to a 21 amino acid sequence (named FD1 for ferroxidase domain 1) within the E2 domain of the protein [159]. The proposition that APP is ferroxidase active has been refuted [160-162], however the conclusion drawn by Duce *et al.* regarding APP's ability to modulate iron efflux from neurons remains sound. Recently, our group has demonstrated that FD1 (now known as FTP, correctly ascribed for its function as a Ferroportin Targeting Peptide) can stimulate hBMVEC ⁵⁹Fe efflux; sAPP exhibited an equivalent activity [162]. This stimulation was not due to iron oxidation by FTP (or sAPP); instead, stimulation of hBMVEC iron efflux by the FTP peptide (and sAPP) occurs via a two-part mechanism. First, FTP binds and stabilizes Fpn in the plasma membrane of hBMVEC. The resulting increase in Fpn in the membrane increases the substrate for subsequent exocytosomal ferroxidase-dependent iron efflux [162]. Both BMVEC and astrocytes express APP, suggesting that this protein might have a role in the regulation of iron transport across the

BBB. Certainly, the fact that APP expression appears to be translationally regulated by iron status *via* the canonical iron regulatory protein1 pathway [163, 164] indicates the likelihood that it does play such a role.

Developmental model of astrocyte-modulated BMVEC iron efflux in the mammalian brain

Iron is used by the brain for myelination, neurotransmitter synthesis, and energy generation. Each of these processes is critical for the proper development of the mammalian brain. Here, we discuss iron transport across the BBB at three points in brain development: 1) postnatal day 0, 2) postnatal day 0-14, and 3) postnatal day 14 and beyond. Specifically, we focus on the export of iron from the basal surface of BMVEC at each point in development of the rat brain with an emphasis on the mechanism(s) of astrocyte-mediated regulation of that export (Fig. 2).

Postnatal day 0

During late embryogenesis BMVEC have formed into blood vessels but astrocytes remain underdeveloped; thus the local microenvironment surrounding BMVEC is devoid of astrocytic end processes (endfeet) [10]. At this stage in development there is an accumulation of iron in BMVEC paired with a lack of transport into the brain parenchyma (Fig. 2A) [31, 165]. In a microenvironment devoid of astrocytes and serum, hBMVEC iron efflux is minimal (Fig. 2A) [14]. Utilizing an *in vitro* reconstruction of this developmental paradigm, the flux of iron from hBMVEC can be manipulated to favor the chamber (apical or basal) containing serum [14]. These data suggest that during embryogenesis, most iron accumulated by BMVEC may be redirected apically back into the serum (blood) from which it came. The lack of BMVEC basolateral iron efflux at these early stages in development may be advantageous for the brain as astrocytes, considered to be the housekeepers of the brain, are severely underdeveloped and are functionally inadequate in handling large amounts of iron [10]. High iron accumulation by the brain would go unchecked, allowing for oxidative damage to neurons.

Postnatal days 0-14

At birth, astrocytes begin to extend their endfeet into the local microenvironment surrounding BMVEC. This process occurs until postnatal day 14 (P14) at which point the astrocytic endfeet make contact with BMVEC [10]. The period between P0 and P14 is marked with a release of BMVEC iron stores as well as an increase in relative brain iron accumulation (Fig. 2B) [30, 31, 165]. Astrocytes which have developed into the local microenvironment surrounding hBMVEC contain the transcript encoding the diffusible ferroxidase sCp [14, 141]. The abundance of the sCp message within astrocytes is increased 4-5 fold when astrocytes are seeded spatially adjacent to hBMVEC [14]. Astrocytes secrete sCp protein into the surrounding media which stimulates iron efflux from hBMVEC *in vitro* [14, 15]. In the absence of hBMVEC Hp, sCp stabilizes hBMVEC Fpn and enhances hBMVEC iron efflux suggesting that sCp catalyzes hBMVEC iron efflux through Fpn [36]. Taken together, the data suggest that communication between BMVEC and astrocytes are crucial for iron transport across the BBB during this developmental juncture.

Also found in the astrocyte secretome is the ferroxidase ferritin [15]. However, it is unlikely that ferritin plays a significant role in hBMVEC iron efflux under non-pathogenic conditions as depletion of sCp from the astrocyte secretome completely abolishes ferroxidase activity from that media resulting in a decreased ability to stimulate hBMVEC iron efflux [14]. *In vivo*, the distal proximity of astrocyte endfeet to BMVEC from P0 through P14 may be advantageous during postnatal ontogenesis. Release of BMVEC iron can be catalyzed and sequestered by astrocytes, which may subsequently transfer that iron to oligodendrocytes for myelination or to neurons for neurotransmitter synthesis. Additionally, the lack of BMVEC-astrocyte contact at this developmental juncture may allow for iron diffusion around astrocytes to nearby oligodendrocytes or neurons.

Postnatal day 14 and beyond

Astrocytes fully envelope and contact the basolateral surface of BMVEC by P14, after which a loss of BMVEC Fpn has been demonstrated in rat brain slices (Fig. 2C) [10, 33]; depletion of BMVEC Hp has also been observed at this stage in development [33]. Utilizing an *in vitro* model BBB system we were able to recapitulate the loss of hBMVEC Fpn by growing hBMVEC and astrocytes in contact with each other [14]. Further analysis revealed that the loss of hBMVEC Fpn is induced by the astrocyte-secreted peptide hormone hepcidin [14]. The CNS of hepcidin knockout mice (Hepc^{-/-}) contain BMVEC that express higher levels of Fpn than wild type mice [166]. Furthermore, injection of hepcidin into the murine CNS induced the turnover of brain Fpn [167]. These data indicate that hepcidin actively decreases the expression of Fpn making it likely that astrocyte-secreted hepcidin can induce BMVEC Fpn turnover *in vivo*.

In regards to function, peak brain iron uptake is reached at P14 after which a sharp decline is observed [30]. Upon contact between astrocytes and hBMVEC, hBMVEC ⁵⁹Fe efflux is significantly diminished [14]. Treatment of our model BBB system with the hepcidin antagonist fursultiamine resulted in restoration of both hBMVEC Fpn protein and iron efflux from hBMVEC. We have demonstrated that hBMVEC iron efflux requires the action of an exocytosomal ferroxidase and Fpn [36]. Thus, we attribute the dramatic decrease in brain iron uptake after P14 to the astrocyte-induced depletion of BMVEC Fpn. The minimal but steady amount of brain iron uptake through BMVEC that occurs during adulthood may be catalyzed by either astrocyte GPI-Cp or other secreted ferroxidases (Fig. 2C).

Prospective model of iron regulation throughout the neurovascular unit of adult mammals

Non-pathogenic model

There is an ever changing presentation of micronutrients to cells of the periphery made possible by the circulatory system. In the brain, an organ which is cut off from the circulatory system, there must be acute adaptations within this system to allow nutrient flux at a moment's notice. We propose that the neurovascular unit (composed of BMVEC, spatially adjacent astrocytes, and neurons) intimately coordinates the flow of ionic iron (not Tf-iron) from one cell to another *via* a form of direct cell-to-cell communication. There is undoubtedly localized communication between the BMVEC and their spatially adjacent

astrocytes [8, 14], and astrocytes in direct contact with both BMVEC and neurons are uniquely positioned to influence neuronal energy metabolism [168] and synaptic function [169]. Furthermore, recent evidence suggests that astrocytes act as a buffer of excess iron, preventing over accumulation by neighboring neurons [170]. We introduce the following model regarding iron regulation throughout the neurovascular unit of the adult mammal.

In periods of iron homeostasis, the metabolic iron requirements of each cell of the neurovascular unit are met (Fig. 3A). Now, assume a situation where neurons are deprived of iron, astrocytes contain iron stores, and BMVEC contain homeostatic levels of iron (Fig. 3B). To restore homeostatic iron levels to neurons, astrocytes secrete iron through Fpn *via* oxidation by GPI-Cp. This iron is then available for incorporation into neurons through DMT1 (or other divalent metal ion transporter, *eg.* Zip8, Zip14) after reduction by an undefined neuronal reductase or by ascorbate secreted by neurons and/or neighboring glial cells (Fig. 3B). This hypothesis is supported through investigations of cellular iron levels in the Belgrade rat model. In the Belgrade rat, which phenotypically has diminished iron acquisition capabilities due to a missense mutation in the gene encoding DMT1, neuronal iron accumulation is diminished [67].

Upon trafficking of iron from astrocytes to neurons, the cytosolic iron levels in astrocytes will drop (Fig. 3C). Astrocyte Fpn will be post-transcriptionally down-regulated under low cytosolic iron levels *via* the binding of IRP to the *Fpn1* IRE (Fig. 3C) [93]; glial cells express IRP which inhibits the translation of Fpn under low iron conditions [34, 105]. An interaction between astrocyte GPI-Cp and Fpn has been demonstrated [47]. Upon depletion of astrocyte Fpn due to low cytosolic iron levels, GPI-Cp loses its interaction with astrocyte Fpn and is now readily available to interact with BMVEC Fpn; BMVEC Hp is depleted in the developed mammalian brain [33]. Through this cell-to-cell protein interaction, iron is sequestered from BMVEC Fpn directly by astrocyte GPI-Cp (Fig. 3D); alternatively, astrocyte sCp may contribute ferroxidase activity to BMVEC Fpn in this scenario. While the interaction between BMVEC Fpn and astrocyte GPI-Cp is a direct mechanism for bringing iron into the brain, one cannot ignore a possible indirect contribution by neurons which may play an intricate role in brain iron uptake as suggested by this model. This inference is suggested by the fact that neurons release glutamate, thus producing calcium waves in astrocytes which travel to their BMVEC-associated endfeet, leading to an increase in the microvascular diameter of brain slices [171]. An increase in microvascular diameter results in increased nutrient supplementation to the brain (*i.e.* to neurons).

Aceruloplasminemia

Aceruloplasminemia is an autosomal recessive disorder caused by loss-of-function mutations in the Cp gene [4, 172, 173]. A defect in the Cp gene will critically affect a neurovascular unit that relies heavily on astrocyte Cp for proper function (Fig. 3). In cases of aceruloplasminemia, astrocytes and neurons contain high levels of iron eventually leading to neuronal loss [4, 12, 174]. Neuronal iron accumulation and death is likely the result of large quantities of iron released from neighboring astrocytes which have undergone cellular necrosis due to iron overload. In the adult model of iron trafficking through the neurovascular unit, astrocyte Cp plays a crucial role in obtaining iron from BMVEC for

astrocytes (Fig. 3D). In the absence of a functioning Cp, it is possible that Hp is up-regulated and supports iron release from BMVEC Fpn. The CNS appears to have evolved compensatory mechanisms for iron oxidation in the absence of either Hp or Cp [120, 135]. Alternatively, ferroxidases in the astrocyte secretome could potentiate the iron mobilization from BMVEC Fpn in scenarios where Cp is inactive. Clearly, these hypotheses remain to be experimentally tested in detail.

Soluble APP and the progression of cerebral amyloid angiopathy

Alzheimer's disease with dementia is characterized by the accumulation of A β peptide aggregates in the vicinity of blood vessels (cerebral amyloid angiopathy) and eventually neurons. These aggregates are the main constituent and potent inducers of cerebral amyloid angiopathy [13, 175]. A β is formed *via* two sequential APP cleavage events (Fig. 4). APP, a transmembrane glycoprotein, is initially cleaved by the β -secretase BACE1 to form a soluble APP product (sAPP) [176-178]. Subsequently, the β -carboxyl-terminal fragment of APP is cleaved by a γ -secretase in the transmembrane region of the protein [176, 178]. The culmination of the sequential cleavage events results in A β peptide, the intracellular C-terminal fragment of APP, and sAPP (Fig. 4).

A β aggregation often occurs in the vicinity of BMVEC prior to the onset of dementia and subsequent Alzheimer's disease [8, 13]. A β peptides catalyze the progression of cerebral amyloid angiopathy and their aggregation is induced by iron [175, 179]. Conformation specific antibodies have been used to discriminate various structural features of A β -metal complexes [179, 180]. Immunological analysis revealed that A β -Fe complexes formed fibrillary oligomers and annular protofibrils, both of which constitute a neurotoxic species [179, 181]. In this context A β may act as a biological reducing agent, reducing Fe^{III} to its more redox-active Fe^{II} state [182]. Reactive iron mediates A β neuronal toxicity *in vitro*, an effect which can be attenuated with iron chelation [183]. Furthermore, A β peptide aggregation is attenuated *via* intranasal administration of the iron chelator deferoxamine [184]. These data suggest that iron plays an intricate role in both the formation and toxicity of A β aggregates.

In the case of cerebral amyloid angiopathy the iron source for A β aggregation may come from BMVEC Fpn. Both sAPP and the synthetic peptide FTP contain the proposed Fpn binding site; the Fpn stabilization that results from the binding of either APP-derived species induces a significant increase in iron efflux from hBMVEC [162]. We hypothesize that sAPP, cleaved from astrocytes, induces brain iron accumulation from BMVEC Fpn. In turn, A β peptides scavenge the incoming iron resulting in protein aggregation leading first to cerebral amyloid angiopathy followed by an eventual neuronal toxicity (Fig. 4).

Future directions

Understanding the mechanisms of iron trafficking into, within, and out of the brain are essential. The potential damaging effects of iron cannot be overlooked as evidence suggests they may be involved in the progression of several neurological disorders. Future experimentation would benefit from a detailed examination of the models proposed in this review. We believe that it is imperative to assess the iron mobilization profile of BMVEC,

astrocytes, and neurons in the context of this neurovascular unit as a whole. Thus, our lab is developing methods to quantitatively dissect the mechanisms of iron transport across the BBB and through the neurovascular unit both *in vitro* and *in vivo*. As a newly appreciated member of the iron regulon [162] APP may be the key to unlocking the mechanisms involved in brain iron accumulation associated with A β aggregation.

For example, the double transgenic mouse model of Alzheimer's disease (APP and presenilin 1, APP/PS1) displays increased expression of the amyloid precursor protein as well as the processing of APP into A β aggregates when fed high iron diets [184]. Amyloid- β aggregates as well as cognitive deficits have been reversed in these mice through intranasal injection of the iron chelating agent deferoxamine (DFO) [184]. Furthermore, DFO inhibits the phosphorylation of tau in the hippocampus of APP/PS1 mice [185]. Accumulation of brain iron in these experiments is likely prevented or suppressed by DFO chelation therapy. The potential exists for a derivative of fursultiamine to be used as a pharmacological agent to prevent or reverse excessive brain iron accumulation as DFO has been demonstrated to do. For example, Fpn is expressed at both the apical and basolateral surfaces of hBMVEC, and is indiscriminant in its ability to direct the export of iron at either surface [14, 36]. Circulating fursultiamine has the potential to stabilize BMVEC Fpn on the apical cell surface, allowing for continuous efflux of iron from BMVEC at the blood side functionally supporting iron efflux from brain. However, along with being lipophilic, fursultiamine has the problem of a short *in vivo* half-life of less than one hour [122]. Therefore, synthesis of a polar fursultiamine derivative with a longer pharmacologic half-life combined with a low iron diet could stabilize Fpn on the apical surface of BMVEC and allow for continuous efflux of iron from the brain. As fursultiamine degrades into thiamine, use of this compound, or a derivative of this compound, may be a safe and effective way of treating Alzheimer's disease. Alternatively, FTP could fulfill this role as well. FTP has the advantage of being recognized as “self” and therefore will not be attacked by the body's immune system. Experiments using FTP to enhance apical iron efflux from hBMVEC are ongoing. The insights gained from these and similar studies should provide new templates for identifying potential drug targets aimed to slow, prevent, or reverse excessive brain iron accumulation.

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Abbreviations

6-OHDA	6-hydroxydopamine
APP	Amyloid- β precursor protein
APPsβ	Soluble β -secretase cleaved APP
Aβ	Amyloid- β
BBB	Blood-brain barrier
BMVEC	Brain microvascular endothelial cells
CNS	Central nervous system
Cp	Ceruloplasmin
CSF	Cerebrospinal fluid
CTFβ	β -carboxyl-terminal fragment
Dcytb	Duodenal cytochrome b
DMT1	Divalent metal transporter 1
E^o	Electrochemical potential
Fpn	Ferroportin
GPI	Glycosylphosphatidylinositol-anchor
hBMVEC	Human brain microvascular endothelial cell line
H-Ferritin	Heavy chain ferritin
Hp	Hephaestin
IRE	Iron-responsive element
IRP	Iron regulatory protein
L-Ferritin	Light chain ferritin
P#	Postnatal day #
sCp	Soluble ceruloplasmin

SDR2	Stromal cell-derived receptor 2
Steap	Six transmembrane epithelial antigen of the prostate
Tf	Transferrin
TfR	Transferrin receptor
Zip8	Zrt/Irt-like protein 8
Zip14	Zrt/Irt-like protein 14
Zp	Zyklopen

Fig. 1. Schematic of plausible iron trafficking mechanisms across a brain microvascular endothelial cell. Mechanism(s) of iron trafficking depicted include transferrin transcytosis, non-canonical iron uptake, or canonical transferrin cycling. In this illustration iron is trafficked from the apical surface (blood side) to the basolateral surface (brain side) of BMVEC. Depicted here, iron is provided either as transferrin (Tf) bound iron (Holo-Tf) or non-Tf-bound iron (NTBI). Tf binds to its receptor, TfR. Ascorbate (Asc) is an example of an extracellular reductant capable of reducing NTBI prior to Fe^{II} transport through the divalent cation transporter [49, 50].

Fig. 2. Illustration diagramming the developmental modulation of BMVEC iron efflux by astrocytes. Graphs are representative of the relative uptake of iron into the brain throughout development. (A) BMVEC iron efflux from Fpn at postnatal day 0 (P0) is dependent upon endogenous Hp. Brain iron uptake at this developmental time point is minimal. (B) BMVEC iron efflux from Fpn at birth through P14 is dependent upon astrocyte-secreted ferroxidases, namely sCp, in addition to BMVEC Hp. Brain iron uptake is abundant during this period of postnatal ontogenesis. (C) Fpn is depleted from BMVEC upon exposure to astrocyte-

secreted hepcidin at postnatal day 14. Iron efflux from BMVEC dramatically decreases after postnatal day 14; a phenotype attributed to the loss of BMVEC Fpn. Note also the depletion of Hp from BMVEC at this developmental juncture.

Fig. 3. Proposed model of the cell-to-cell modulation of iron trafficking within the adult neurovascular unit. (A) Homeostatic levels of intracellular iron in BMVEC, astrocytes, and neurons. (B) Astrocytes supply iron to neurons deficient in iron. (C) Neuronal iron levels are restored. Intracellular levels of iron within astrocytes drop after sufficiently supplying neurons. This drop in astrocyte iron causes the binding of endogenous IRP to the IRE of Fpn inhibiting translation. Depression in iron efflux through astrocyte Fpn catalyzes turnover of the permease. (D) Depletion of Fpn from the plasma membrane of astrocytes allows for

GPI-Cp to catalyze iron efflux from BMVEC Fpn which can then be sequestered by astrocytes. This sequestration of iron from BMVEC will restore the intracellular iron levels of astrocytes allowing the cycle to continue. Iron is depicted entering cells through a divalent cation transporter represented here with DMT1.

Fig. 4.

Formation of soluble APP and its role in the progression of cerebral amyloid angiopathy. The astrocyte full length transmembrane glycoprotein APP is cleaved in two sequential steps. Initially, β-secretase (BACE1) cleaves the protein followed by γ-secretase cleavage of the remaining β-carboxyl-terminal fragment (CTFβ) to form amyloid-β (Aβ) peptide. The β-secretase cleaved soluble APP (sAPP) containing the FTP sequence induces iron efflux from BMVEC Fpn. Iron released from BMVEC Fpn triggers the aggregation of Aβ peptides in the vicinity of the blood vessels leading to the progression of cerebral amyloid angiopathy and subsequent dementia.

Table 1
Key proteins involved in cellular iron uptake

Gene Name	Protein	IRE*	Function	Brain Localization
<i>TF</i>	Transferrin	-	Iron chaperone	Choroid plexus (lower mammals only) [186], oligodendrocytes [20], astrocytes [22], neurons [21]
<i>TFRC</i>	Transferrin Receptor 1	3'-UTR	Iron acquisition	Choroid plexus [187], cultured astrocytes [188], cultured oligodendrocytes [189], neurons [190, 191], BMVEC [28, 30, 33, 40, 187]
<i>STEAP2</i>	Steap2	-	Ferric reductase	BMVEC [40]
<i>CYBRD1</i>	Dcytb	-	Ferric reductase	BMVEC [40], astrocytes [46-48]
<i>DMT1/DCT1/NRAMP2/SLC11A2</i>	DMT1	3'-UTR	Ferrous permease (import)	BMVEC [29, 34, 40], neurons, oligodendrocytes, ependymal cells [29, 67]
<i>SLC39A8</i>	Zrt/Irt-like protein 8		Ferrous permease (import)	Brain (cellular distribution unknown) [72]
<i>SLC39A14</i>	Zrt/Irt-like protein 14		Ferrous permease (import)	Brain (cellular distribution unknown) [72]
<i>FTH1</i>	Ferritin heavy chain	5'-UTR	Iron oxidase	Oligodendrocytes [1], astrocytes [15, 155], microglia [1], pyramidal neurons [1]
<i>FTL</i>	Ferritin light chain	5'-UTR	Iron oxidase/storage	See <i>H-ferritin</i>

* Iron regulatory protein (IRP) binds the iron-responsive element (IRE) under low cellular iron conditions preventing nucleolytic turnover (3'-UTR) or translation initiation of the transcript (5'-UTR).

Table 2
Kinetics of BMVEC and divalent cation transporters for select metal ions

Divalent Cation	hBMVEC IC ₅₀	hBMVEC K _{0.5} /I	DMT1 K _M	ZIP8 K _M	ZIP14A/B K _M
Fe ^{II}	-	3.9 ± 1.1 μM [40]	1.5-3 μM [58]	0.7 μM [73]	2.0 μM [70]
Mn ^{II}	8.4 ± 2.5 μM [40]	8.4 μM *	1-2 μM [58]	2.2 μM [192]	4 μM (B); 18 μM (A) [72]
Zn ^{II}	3.1 ± 0.7 μM [40]	3.1 μM *	42 μM [193]	0.26 ± 0.09 μM [194]	2.0 μM [70]

* Application of the Cheng-Prusoff equation [195] to respective IC₅₀ values provided estimated K_I values.

Table 3
Key proteins involved in cellular iron efflux

Gene Name	Protein	IRE*	Function	Brain Localization
<i>FPN1/REG1/MTP1/SLC40A1</i>	Ferroportin	5'-UTR	Ferrous permease (export)	BMVEC [14, 33, 36, 116, 121], neurons [116, 118, 119], oligodendrocytes [116, 118-120], astrocytes [47, 116], ependymal cells [116, 118]
<i>HEPH</i>	Hephaestin	-	Iron oxidase (export)	Astrocytes, oligodendrocytes, microglia, neurons [134], BMVEC [33, 36, 135]
<i>CP</i>	Ceruloplasmin	-	Iron oxidase (export)	Astrocytes [14, 15, 140], ependymal cells [137], oligodendrocytes [120], BMVEC [36]
<i>HEPHL1</i>	Zyklopen	-	Iron oxidase (export)	Brain (cellular distribution unknown) [128]
<i>FTH1</i>	Ferritin heavy chain	5'-UTR	Iron oxidase (export)	Oligodendrocytes [1], astrocytes [15, 155], microglia [1], pyramidal neurons [1]
<i>FTL</i>	Ferritin light chain	5'-UTR	Iron oxidase/storage	<i>See H-ferritin</i>
<i>APP</i>	Aβ precursor protein	5'-UTR	Iron oxidase (export)	BMVEC [158], neurons [145], astrocytes [13, 145]

* Iron regulatory protein (IRP) binds the iron-responsive element (IRE) under low cellular iron conditions preventing nucleolytic turnover (3'-UTR) or translation initiation of the transcript (5'-UTR).