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The Blood Brain Barrier — Regulation of Fatty Acid and Drug Transport

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1. Introduction

1.1. The blood brain barrier

The blood brain barrier (BBB) is a selectively permeable cellular boundary between the brain and the peripheral circulation. The principal component of the BBB is the capillary or micro-vessel endothelial cell (Figure 1). The endothelial cells in the brain capillaries differ from those in the peripheral vasculature in several key features:

1. Presence of tight junctions (TJ) that limit the paracellular passage of macromolecules.
2. Restricted rate of fluid-phase endocytosis that limits the transcellular passage of macromolecules [1]
3. Presence of specific transporter and carrier molecules [2]
4. Lack of fenestrations [3]
5. Increased mitochondrial content [3]

Thus, the endothelial cells of the BBB are less “leaky” than those of the peripheral vessels. However, it has been shown that if the endothelial cells of the brain capillaries are removed from their natural environment and allowed to vascularize the peripheral tissue, they become more leaky [1]. In contrast, the endothelial cells from the periphery form tight junctions when allowed to vascularize the brain parenchyma. Morphologically, the tight junctions of the BBB

Figure 1. Neurovascular Unit of the blood brain barrier consists of the endothelial cells (pink) surrounded by basement membrane (gray), pericytes (yellow) and astrocyte foot processes. The tight junctions (black lines) formed between two endothelial cells restrict the paracellular diffusion of compounds.

resemble the tight junctions between epithelial cells rather than those between peripheral vascular endothelial cells [4].

The unique tight junctions of the BBB are responsible for producing very high transendothelial electrical resistance (TEER) of $1500 - 2000 \Omega\text{cm}^2$ [2,5,6]. Though the microvessel endothelial cells play a primary role in the formation of the BBB, several other cells are equally important in maintaining the integrity of the BBB. These cells, namely, the astrocytes, pericytes, neurons and other glial cells are said to form a “neurovascular unit” [7]. Integrity of the BBB is of utmost importance in maintaining the homeostasis of the brain microenvironment. Disruption of the BBB is seen in various states of inflammation (multiple sclerosis), neoplasia, infections (meningitis, encephalitis), trauma and Alzheimer disease [8,9]. It would be highly desirable to develop therapeutic strategies to reverse this disruption and tighten the BBB. At the same time, a transient opening of the BBB would be advantageous for delivery of drugs into the brain in conditions like epilepsy or Parkinson disease [2].

1.2. Functions of the BBB

The BBB is responsible for maintaining the appropriate ionic composition of the interstitial fluid of the brain that is required for optimum functioning of the neurons. To achieve this, the BBB functions as a *transport barrier* by facilitating the uptake of the required nutrients, while preventing the uptake of, or actively effluxing certain other molecules or toxic by-products of metabolism [10]. The BBB also functions as a *metabolic barrier* by virtue of possessing intracellular and extracellular enzymes. For example, extracellular enzymes such as peptidases and nucleotidases break down peptides and ATP, respectively. Intracellular enzymes like cytochrome P450 (CYP450), primarily CYP1A and CYP2B degrade noxious substances and prevent their entry into the brain parenchyma [10].

1.3. Role of astrocytes in the BBB

It is now known that the astrocytes play a key role in the conditioning and development of the brain microvessel endothelial cells (BMEC). Astrocytes are one of the glial cells of the central nervous system (CNS) that play several important roles in the structure and function of the CNS. They are intimately associated with the BMEC such that their foot processes ensheath 99% of the external surface of the BMEC [11]. Astrocytes have been shown to alter the properties of cocultured brain endothelial cells in the following ways [11,12].

1. Increase in barrier-related marker enzyme activities, such as that of γ -glutamyl transpeptidase (GGT) and alkaline phosphatase.
2. Enhanced expression of a glucose transporter.
3. Elevation of trans-endothelial electrical resistance (TEER).
4. Tightening of the BBB as seen by decreased paracellular permeability of sucrose.
5. Increase in tight junction number, length and complexity.

It has also been shown that BMEC monolayers are less leaky if grown in the presence of astrocyte-conditioned medium (ACM) [1,11]. The precise molecular nature of the astrocyte-derived factors that is responsible for the tightness of the BBB have yet to be unequivocally elucidated. However, several factors have been postulated to play a role including glial cell-derived neurotrophic factor (GDNF), transforming growth factor-beta (TGF- β), and src-suppressed C-kinase substrate (SSeCKS) that leads to increased angiopoietin-1 secretion. The BMEC themselves are known to secrete factors that help in the maintenance of astrocyte health. One such putative factor is the leukemia-inhibitory factor (LIF), a cytokine known to be involved in astrocyte differentiation [11].

1.4. Role of pericytes in the BBB

The pericytes are specialized cells of mesenchymal lineage that have multiple organ-specific roles. For example, they are present in the kidney as mesangial cells, in the liver as perisinusoidal stellate cells and in the bone as osteoblasts [13,14]. The pericytes in the central nervous system are closely associated with the BMEC and play an important role in the maintenance of the BBB. Their functions include [14].

1. Cerebrovascular autoregulation and blood flow distribution
2. Differentiation of the BBB
3. Formation and maintenance of the tight junctions of the BBB.
4. Initiation of the extrinsic (tissue factor) pathway of blood coagulation following cerebrovascular injury
5. Brain angiogenesis via secretion of angiopoietin-1
6. Phagocytic and scavenging (macrophage-like) functions
7. Production of immunoregulatory cytokines like IL-1 β , IL-6 and GM-CSF
8. Regulation of leukocyte transmigration, antigen presentation and T-cell activation.

2. Molecular components of the tight junctions

The tight junctions consist of both membrane proteins as well as cytoplasmic proteins [15] (Figure 2). The integral membrane proteins are Claudins, Occludin and Junctional adhesion molecules (JAM). There are also several cytoplasmic accessory proteins that form a plaque and function as adapter proteins to link the membrane proteins to the actin cytoskeleton of the cell [16,17]. These include Zonula occludens proteins (ZO-1, ZO-2, ZO-3), Cingulin, AF-6, 7H6 antigen and Symplekin. These tight junctional complexes are not static structures but rather very dynamic entities that can “bend without breaking”, thereby maintaining structural integrity [8].

2.1. Claudins

The claudins are a large family of transmembrane phosphoproteins [15]. Twenty-four members have been characterized so far, claudins 1-24 [18,19]. Of these, claudins 1, 3, 5 and 12 have been shown to form the tight junctions of the BBB [9,17,20,21]. Claudin-5 appears to be specific to the tight junctions of the endothelial cells and is called the “endothelial claudin” [17]. Each claudin molecule has 4 transmembrane domains. The claudin on one cell binds homotypically to the claudin on the adjacent cell to form the seal of the tight junction. The claudins, along with occludin and the JAMs, form the tight Junctional strands that keep the cells together and prevent paracellular flux of macromolecules from the apical to the basolateral side of polarized cells like BMEC [18]. The cytoplasmic carboxy terminal of the claudins binds to the cytoplasmic ZO proteins [20]. Claudin-1 is an integral component of the tight junctions and its loss is associated with certain pathologic conditions like tumours, strokes and inflammatory diseases [21].

2.2. Occludin

Occludin is a 65-kDa transmembrane phosphoprotein and is distinct from the claudins. However, its subcellular localization parallels that of claudins and, like the claudins, it has

Figure 2. Schematic representation of proteins that are involved in the formation of the tight junction and adherens junctions in brain microvessel endothelial cells.

four transmembrane domains. The expression of occludin is higher in the adult BMEC compared to the peripheral endothelial cells. However, it is not expressed in the fetal or newborn human brain. Occludin plays an important structural, as well as a functional, role in the regulation of BBB permeability. As is the case with several other tight junction-associated proteins, phosphorylation or dephosphorylation of serine, threonine or tyrosine residues on the occludin molecule is crucial for its proper functioning [17,18,22,23]. For example, phosphorylation of occludin at serine and tyrosine residues correlates with tight junction assembly or tightening [8].

Occludin and the claudins interact intricately on the BMEC membrane. Together, they form channels that tightly regulate the paracellular flow of ions and other hydrophilic molecules. Thus, they are both essential in the formation, maintenance and regulation of the BBB [16,18].

2.3. Junctional Adhesion Molecules (JAM)

These molecules play an important role in the regulation of tight junction permeability in endothelial and epithelial cells [24]. These glycoproteins are members of the immunoglobulin superfamily of proteins. Three different JAMs have been characterized in humans, JAM-1,

JAM-2 and JAM-3, also referred to as JAM-A, JAM-B and JAM-C, respectively. Besides endothelial and epithelial cells, these molecules are also found on the surface of erythrocytes, leukocytes and platelets and are thought to contribute to various processes like leukocyte migration, platelet activation, angiogenesis and binding of reovirus [25]. The JAMs have short cytoplasmic tails that interact with cytoplasmic accessory proteins like ZO-1 and may require activation by phosphorylation, mediated by certain atypical protein kinases.

2.4. Cytoplasmic accessory proteins

Several cytoplasmic proteins appear to be essential components of the tight junctions. Among them, the zonula occludens proteins (ZO-1, ZO-2, ZO-3) play an important role. These 3 proteins have a molecular mass of 220, 160 and 130 kDa, respectively. They belong to a family of proteins called MAGUK (membrane-associated guanylate kinase-like protein) and form the submembranous plaque of the tight junction [2,15]. They are structurally complex proteins with several domains that make direct contact with claudins, occludin and JAM on one side and the actin cytoskeleton on the other [15]. Cingulin is a double-stranded myosin-like protein that serves as scaffolding and links the TJ accessory proteins with the cytoskeleton [8]. Actin, the cytoskeletal protein, plays a central role in the maintenance of the TJ. Actin-degrading macromolecules, such as cytochalasin-D, phalloidin and certain cytokines lead to disruption of the actin cytoskeleton and hence, of the tight junctions [8].

The tight junctional proteins can be modulated by several intracellular processes that involve calcium-signaling, phosphorylation, G-proteins, proteases and by TNF- α [4,8]. The tight junctional complexes also help localize the proteins and lipids of the apical and basolateral cell membranes in their respective compartments and prevent free mixing of these cell membrane macromolecules between the two domains. Thus, the BMEC owe much of their polarity to the TJ complexes [2,26].

3. Regulation of BBB permeability

Various factors play a role in regulating the permeability of the BBB as follows [2]:

1. Post-translational modifications of the TJ proteins. For example, phosphorylation and dephosphorylation mediated by protein kinases and phosphatases, respectively.
2. Alteration of the actin cytoskeleton.
3. Proteolytic degradation of certain TJ components like occludin, mediated by metalloproteinases.

4. *In vitro* models to study the BBB

In vitro models of the BBB have proven very effective to study the transport of endogenous macromolecules like fatty acids across the BMEC. They have also been used extensively in

pharmaceutical research to study the passage of therapeutic molecules across the BMEC [5-7]. Several studies have shown that the BMEC lose many of their special properties when removed from their natural environment and show “dedifferentiation” behaviour. Thus, one potential limitation of in vitro BBB models is that the BMEC may not behave as site-specific specialized endothelial cells in vitro, but rather as common peripheral endothelial cells [7]. In spite of this shortcoming, several successful in vitro models of the BBB have been described [27]. Many of these have used human, bovine, and porcine or rat endothelial cells:

1. Alone [5,6,28-30], or
2. in combination with astrocyte conditioned medium supplemented with agents that elevate intracellular cAMP [1], or
3. Co-culture of endothelial cells on one side of a filter, with astrocytes on the other [31].

5. FA transport across the BBB and effects of FA on BBB permeability

Fatty acids (FA) are key components of membranes and exhibit many biological functions in a variety of tissues, including the key energy source for mitochondrial β -oxidation [32,33]. Cells acquire fatty acids through *de novo* synthesis, hydrolysis of triglycerides (TG) or uptake from exogenous sources [33]. Minimal amount of FA are derived from TG hydrolysis and most cells are dependent upon fatty acid uptake from the peripheral blood [32,34]. FA from the diet are absorbed by enterocytes in the small intestine and packaged into chylomicrons as TG. The liver also produces very low density lipoprotein (VLDL), a rich source of endogenously generated TG. Circulating chylomicrons and VLDL particles are hydrolyzed by lipoprotein lipase in the capillary lumen of tissues and the released FA from these lipoproteins may be taken up by tissues in the body [35]. FA that enter into cells are then esterified and stored as TG or transported to the mitochondria for β -oxidation. The importance of FA for the developing and adult brain has been recently reviewed [6]. FA transport from blood into parenchymal neurons is much more difficult than other cells since the tight junctions of the BBB severely restrict passage into the brain. FA must first move via transcellular transport across both the luminal (apical) and abluminal (basolateral) membranes of the endothelial cells and then across the plasma membrane of the neural cells [36-38].

The mechanism of FA transport into the brain remains controversial. Several studies support the notion that FA can move across membranes by diffusion [39,40]. Alternatively, others studies indicate that FA may enter into cells via specific protein-mediated transport [32,41,42]. In the diffusion model, once bound to the outer membrane leaflet, they quickly reach ionization equilibrium and the non-ionized form of fatty acids move across the membrane more rapidly than the ionized form [43]. The main problem with the FA diffusion model has always been whether diffusion is rapid enough to supply cells, which have a high long-chain FA metabolic requirement with sufficient amount of FA for β -oxidation [44]. In the protein-mediated transport model selective transport of FA occurs via specific protein transporters found on the cell membrane [33,41,45-47]. The mechanism of FA transport into the brain and the involve-

ment of FA protein transporters has been reviewed [6]. We recently showed that the transport of various FA across confluent layers of HBMEC was, in part, mediated by fatty acid transport proteins (FATPs) [5,6]. Knock down of FATP-1 and CD36 resulted in reduced FA transport. In addition, transport appeared to be dependent upon fatty acyl chain length and degree of unsaturation.

The role of FA, such as arachidonic acid (AA), on BBB permeability is well documented and controversial. Studies have indicated that a rapid influx of AA into the brain occurs upon plasma infusion with AA [48,49]. In addition, a permeability-enhancing and neurotoxic effect of AA has been observed [50-52]. AA is a precursor for the formation of various bioactive molecules including prostaglandins, such as PGE₂, and leukotrienes. Several studies have indicated that the increase in BBB permeability is correlated with the formation of PGE₂ [29,30, 53-56]. The prostaglandin EP2 receptor was shown to be responsible for mediating the neuroinflammatory and neurodegenerative effects of PGE₂ in a mouse model of status epilepticus [57]. The permeability increase caused by AA in pial microvessels of rats was effectively blocked by a combination of indomethacin (COX inhibitor) and nordihydroguaric acid (LOX inhibitor) but not singly by either agent [58]. In that same study, AA-mediated permeability increase was blocked by superoxide dismutase and catalase. These authors concluded that free radicals generated by either COX or LOX pathways were responsible for the permeability response to AA. In a mouse model of diabetic retinopathy 12-HETE and 15-HETE, products of the lipoxygenase pathway, were shown to be responsible for increasing the permeability of retinal endothelial cell barrier via an NADPH oxidase-dependent mechanism [59]. Interestingly, AA inhibited the cytokine-induced up-regulation of several genes involved in endothelial cell inflammation [60].

However, other studies have suggested that AA metabolites, such as PGE₂, have a protective role in the microvessels of the CNS and that PGE₂ prevents permeability increases. For example, the permeability increase caused by bradykinin was prevented or attenuated by exogenously added PGE₂ and iloprost, a prostacyclin analog [61]. In that study, COX-inhibitor drugs potentiated the permeability increases caused by bradykinin, thus suggesting an inhibitory role of PGE₂ in increasing endothelial cell permeability. In addition, PGE₂, acting via EP4 receptors, inhibited the increase in BBB permeability in a mouse model of experimental autoimmune encephalomyelitis [62]. Moreover, PGE₂, acting via EP2 receptors, has neuroprotective properties and limits ischemic damage in mice stroke models [63]. It has been postulated in these studies [61,62] that engagement of EP2 and EP4 receptors by PGE₂ leads to an increase in cAMP levels. This cAMP accumulation has been shown to potentiate cadherin-mediated cell-cell contact and enhance endothelial barrier function. Thus, PGE₂ may promote BBB integrity via direct action on endothelial cells [62].

Several studies have demonstrated that microvessel endothelial cells from various organs have the capacity to produce a range of eicosanoids, notably, PGE₂, PGI₂ and PGF_{2α}. In most of these studies the endothelial cells were stimulated with the calcium ionophore A23187 in addition to exogenously added AA [64-66]. However, in one study endothelial cells exposed to plasma from preeclamptic women showed increased production of prostaglandins [67]. In addition,

bovine brain microvessel endothelial cells (BBMEC) exposed to TNF- α released large amounts of PGE₂ over a 12-hour period [29].

Previous work has shown that docosahexanoic acid (DHA) is converted to its vasodilator metabolite, 17S-HDoHE in endothelial cells [68]. DHA is a precursor in the formation of several bioactive molecules in human blood cells and in glial cells [69]. However, in those experiments, the cells were exposed to stimulants like zymosan A or the calcium ionophore, A23187 to facilitate the release of DHA metabolites. These metabolites have been shown to have several biological effects like inhibition of inflammation and platelet aggregation, mediation of vasodilation, anti-arrhythmic effects and lowering of triglyceride levels [70].

6. Drug transport across the BBB

The tight junction complex that connects brain microvascular endothelial cells in the BBB as well as the epithelial cells of the choroid plexus that form the blood-cerebral spinal fluid barrier (BCSFB) serve as a physical barrier preventing the paracellular diffusion of endogenous and exogenous compounds. The presence of these tight junctions is essential for maintaining the proper environment required for neuronal transmission. However, paracellular diffusion of nutrients and metabolites between the blood and the extracellular compartment of the brain is also highly restricted. Consequently, the uptake of essential molecules, such as glucose and amino acids, to meet the metabolic requirements of the brain occurs through specific transporter proteins located on the plasma membrane of the endothelial cells. In addition to transporters that facilitate the entry of various solutes into the brain, the brain endothelial cells also express numerous efflux transporters [71]. These transporters are members of the ATP-binding cassette (ABC) protein family and utilize energy from adenosine triphosphate (ATP) hydrolysis to actively remove compounds from the cells against a concentration gradient.

From a drug transport perspective, there are several transporters that are critically involved in the movements of drugs across the BBB. These include organic anion-transporting polypeptide 1A2 (OATP1A2/SLO1A2), organic anion transporter 3 (OAT3/SLC22A8), monocarboxylate transporter 1 (MCT1/SLC16A1), from the solute transporter family, and P-glycoprotein (P-gp; MDR1/ABCB1), breast-cancer-resistance protein (BCRP/ABCG2) and multidrug-resistance-associated proteins 1-9 (MRP1-9/ABCC1-9) from the ABC transporter family [72]. The localization of these transporters in both the BBB and BCSF barrier are shown in Figure 3 with each individual transporter is being discussed in greater detail below.

6.1. Organic Anion Transporting Polypeptide (OATP)

Organic anion transporting polypeptides (OATPs) are members of the solute carrier organic anion transporter family (SLCO) [73]. The OATPs accommodate the transport of a wide variety of amphipathic solutes, including bile salts, anionic peptides, steroid conjugates, thyroid hormones and an increasing number of pharmaceutical drugs and xenobiotics [74]. Members of the OATP family, of which there are currently 11 known to be expressed in humans

Figure 3. The localization of transporters in the blood brain barrier (BBB) and blood cerebral spinal fluid barrier (BCSFB) of CNS.

(OATP1A2, 1B1, 1B3, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1, 5A1, and 6A1), share a great deal of amino acid sequence identity and transport solutes in a sodium independent manner [75].

Of the various OATPs, both OATP1A2 and OATP1A4 are expressed in the BBB. Organic anion transport protein 1A2 was the first member of the OATP family to be reported in humans, while OATP1A4 is a more recently discovered homolog of hepatic [76]. At the protein level, OATP1A2 (previously designated OATP-A) is expressed in many organs including the liver, intestine, kidney, lung, testes, and the brain. Within the brain, this transporter is localized in the frontal cortex and specifically confined to the endothelial cells of the BBB [74]. Its localization on the luminal side of brain microvessel endothelial cells suggests that OATP1A2 aids in the entry of various solutes and therapeutic agents into the brain [74]. While OATP1A4 is mainly concentrated in the liver, the transporter has also been detected within the brain microvessel endothelial cells [74]. In contrast to OATP1A2, immunohistochemical localization studies indicate that OATP1A4 is expressed on both the apical and basolateral side of the endothelial cells of the BBB and thus, mediates the uptake of compounds from both the brain and the blood compartments [77].

6.2. Organic Anion Transporter (OAT)

Organic anion transporters (OATs) belong to the SLC22A gene family. Similarly to OATPs, the OATs transport a broad range of chemically unrelated endogenous and exogenous compounds. There are at least 10 families of OATs designated by Arabic numbers (eg. OAT1).

OAT1 is predominantly expressed in the kidney although a very small amount is also found in the brain particularly concentrated in regions such as cortex, hypothalamus, hippocampus and cerebellum [78]. This transporter is known to interact with a broad range of drugs including antibiotics (penicillins, benzylpenicillin and carbenicillin), antineoplastics (methotrexate) and even cholesterol lowering drugs including the statins and fibrates such as fluvastatin, pravastatin, and bezafibrate, respectively [78]. OAT2, on the other hand, is predominantly expressed in the liver and very little is found in the kidney and brain. The expression level of this transporter in a particular tissue can be influenced by a variety of factors; including gender and species differences [79]. For example, in the adult male rat, the mRNA for OAT2 expression is greater in the liver than the kidney, and the opposite is true for the adult female rat where the mRNA level in the kidney is greater than in the liver [79]. However, this phenomenon has not been observed in humans. Furthermore, the expression level of OAT2 is also influenced by hepatocyte nuclear factors and endogenous gas molecules including nitric oxide [80,81]. Given the similar molecular structure to OAT1, OAT2 also mediates the transport of a broad range of solutes including cholesterol lowering drugs (i.e. statins), antibiotics such as cephalosporins, and antineoplastic drugs like 5-fluorouracil [78].

From a CNS perspective, OAT3 appears to have the greatest expression levels in the brain [78]. Within the CNS, OAT3 is primarily localized in the brain capillaries and in epithelial cells of the choroid plexus, specifically on the basolateral side of the plasma membrane of the cells [82]. The predominantly basolateral localization of OAT3 in the BBB and BCSFB implies that the primary function of OAT3 is to aid in the removal of compounds from the brain. Endogenous products of neurotransmitter and hormone metabolism are potential candidates for OAT3-mediated removal. Potential therapeutic agents that may be transported out of the brain through OAT3-dependent processes at the BBB and BCSFB include the various statins, diuretics, antibiotics and antivirals [78]. As OAT3 interacts with a large number of therapeutic agents, drug-drug interactions may be of potential concern in the BBB, although specific examples are at present not known.

6.3. Glucose Transporters (GLUT)

Glucose is the major source of energy for most mammalian cells, particularly in the brain. Despite the high dependence of the brain on glycolysis, the source of glucose comes entirely from the blood and is dependent on passage through the BBB. The entry of glucose into the brain is mediated by facilitative glucose transporter proteins. There are currently seven known isoforms, with the designation of GLUT1-7 [83]. The main isoforms found within the CNS are GLUT1 and GLUT3 that bring glucose into the cell through sodium independent transport mechanisms. A summary of the various GLUTs and their distribution within the CNS is shown in Figure 4. GLUT1 within the CNS exists as two distinct forms, which differ only by the extent of glycosylation [84]. A glycosylated, 55 KDa GLUT1 is found primarily in the endothelial cells of the BBB while the non-vascular, non-glycosylated 45 KDa form is mainly found in neural cells as well as the basolateral plasma of epithelial cells isolated from the choroid plexus [83]. Aside from the prominent expression found in the microvessels and choroid plexus, GLUT1 has also been detected in small cells with dark stained nuclei characteristic of glia cells [85].

Immunohistochemistry staining also showed a positive detection of GLUT1 in astrocytes that are in direct contact with the cerebral microvessels of rat brain slices. Electron microscopy also revealed dense distribution of GLUT1 within the astrocyte foot processes surrounding the microvessels of the gray matter and synaptic contacts [86].

Figure 4. Cellular localization of different isoforms of glucose transporter in the CNS.

The main glucose transporter in the BBB is GLUT1. This same transporter is also highly expressed in the blood-retinal barrier, the placental barrier, and blood-CSF barrier (BCSFB) [87-89] highlighting its importance in regulation of glucose levels in these tissues. The transport of glucose through GLUT1 in the BBB is the rate-limiting step for glucose utilization in the brain and is highly responsive to metabolic changes within the brain. For example, GLUT1 expression in the BBB at both the mRNA and protein level can increase or decrease depending on the ambient concentration of hexose. High concentration of hexose decreases the expression of GLUT1 while low hexose concentration causes an up-regulation of both GLUT1 mRNA and protein levels [90]. Following brain injury such as a stroke and brain tumors, both mRNA and protein levels of GLUT1 are significantly increased [83,85,91].

The endothelial cells forming the BBB also express sodium glucose cotransporter (SGLT) [92]. Unlike GLUT1, glucose transport through SGLT is sodium-dependent. A functional role for SGLT in glucose homeostasis in the brain has not been established; however, it has been

speculated that SGLT may help maintain intracellular glucose levels in the brain under stressful conditions such as hypoglycemia [92].

6.4. Monocarboxylate Transporter (MCT)

Monocarboxylic acids, including lactate, pyruvate, and ketones play an important role in energy metabolism within the body. Monocarboxylates such as pyruvate, lactate, and ketone bodies (i.e. acetoacetate and β -hydroxybutyrate) can be utilized by neurons, in the absence of glucose, to generate a substantial amount of energy for the brain [93]. Under pathological and physiological conditions including diabetes, prolonged starvation, hypoglycemia, or even intense exercise, the build-up of lactate provides an energy source, which can be utilized by the brain [3,94,95]. In addition, monocarboxylates including lactic acid are a metabolic by-product produced and released within the CNS by neurons [96,97], astrocytes [96] and oligodendrocytes [98]. As monocarboxylates are hydrophilic compounds that cannot readily diffuse cross the BBB, specific transporter systems are required to maintain proper levels of these endogenous metabolic products in the brain [93].

Sequence homology indicates that the monocarboxylate transporter family (previous known as SLC16 gene family) consists of 14 members identified as MCT1-9, MCT11-14 and T-type amino acid transporter 1 (TAT1) [99]. MCT1-4 is a symporter mediating the co-transport of monocarboxylate and proton in a one to one stoichiometry ratio. MCT1-4 is present in almost all tissues including the muscles, liver, kidney, heart, testes, and brain [93,99]. While MCT1 and MCT2 are found in the muscles, liver, kidney, heart and CNS, [93], MCT3 is exclusively expressed on the basolateral side of the retinal pigment epithelium and MCT4 is highly expressed in the skeletal muscles and also in the brain. Within the BBB, MCT1 was the first monocarboxylic acid transporter identified in the brain microvessel endothelial cells and in the ependocytes lining the ventricles [100]. Both electron microscopy and immunohistochemistry revealed a small amount of MCT1 in astrocytic end-feet surrounding the capillaries [100,101]. The presence of MCT1 was found in the cytoplasm of astrocyte and also associated with the plasma membrane [93]. In contrast, MCT2 is found in endothelial cells forming the BBB, but absent in astrocytes [93,100]. MCT4, on the other hand, was exclusively expressed in the astrocytes and glial cells of rodent brain. Furthermore, when the hippocampus and the corpus callosum were labeled, the expression of MCT4 was restricted to astrocytes [93]. MCT8 was recently recognized as thyroid hormone transporter as opposed to monocarboxylate [102].

6.5. ATP-Binding Cassette Transporters (ABC)

The ATP-binding cassette (ABC) superfamily of transporter proteins are responsible for the active transport of a wide variety of compounds including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, bile acids, drugs and other xenobiotic compounds across cellular membranes. There are roughly 48 genes encoding the various human ABC transporters, each is organized into seven subfamilies designated ABCA to ABCG [103]. Over-expression of ABC transporters are major contributors to the development of multidrug resistance (MDR) in cancer cells. For instance, when the MDR gene that codes for an efflux transporter is being transfected into drug sensitive cells, the transfectant cells become

resistant to the drugs that are substrates for the transporters resulting in a decrease in the intracellular concentration of the drugs, thereby conferring multidrug resistance [104]. MDR in tumor cell lines is often linked to an ATP-dependent decrease in cellular accumulation of drugs namely through p-glycoprotein (p-gp encoded by ABCB1), multidrug resistance proteins (MRP encoded by ABCC), and breast cancer resistance protein (BCRP encoded by ABCG2) drug efflux transporters [105]. In addition to their function as multidrug resistance proteins, these transporters are also expressed in normal tissue such as intestines, liver, kidney and the BBB and BCSFB, suggesting that they also have a protective function in limiting accumulation and distribution and speeding the elimination of xenobiotic compounds which could result in tissue toxicity [106].

6.6. Multidrug Resistance Protein (MRP)

The multidrug resistance-associated proteins (MRPs) are a subfamily of ABC transporters. There are currently 12 members of this subfamily designated as ABCC1-12. Of the 12, 9 have demonstrated drug efflux transporter function and play an important role in absorption, distribution and elimination of various drugs and metabolites. While all MRPs have the capability to transport amphiphatic organic anions, transport substrates are not limited to anionic species. Examples of this include the transport of nucleotide based analogs by MRP4 and MRP5, efflux of prostaglandins by MRP1, co-transport of neutral or cationic solutes as well as glucoronide drug conjugates by MRP1 and MRP2 [107]. Within the MRPs and other ABC transport proteins there tends to be substantial substrate affinity overlap. This is a fascinating feature considering most members are structurally and functionally distinct from other ABC binding cassette transporters. For example, there is only approximately 15% amino acid sequence homology between MRPs and P-gp [108]. In addition, when comparing amino acid sequence between different members within the MRP subfamily to MRP1, amino acid sequence homology ranges between 33% for MRP8 and 58% for MRP3 [109].

The brain endothelial cells that form the BBB express several different MRP. Collectively the MRP efflux transporters function to restrict the uptake and aid in the elimination of drugs, xenobiotics and endogenous compounds from the brain. Currently, members of the MRP family that have been reported in the BBB include MRP1, 2,4-9. The evidence for the localization and function of each of the MRPs within the BBB are discussed below.

6.6.1. MRP1

MRP1 is expressed in primary cultured bovine, murine [110], rat [111] and porcine [112] brain microvessel endothelial cells. While studies by Seetharaman and coworkers [113] suggested up-regulation of MRP1 expression in human culture brain microvessel endothelial cells compared to freshly isolated human brain capillaries, more recent studies support robust expression of MRP1 within the brain capillaries isolated from human brain tissue [114]. Two independent studies reported that MRP1 is localized primarily to the apical (luminal) plasma membrane in brain microvessel endothelial cells [114,115]. This is in contrast to studies by Roberts et al. [116] suggesting MRP1 has a basolateral (abluminal) plasma membrane localization in rat brain microvessels. As MRP1 shows high transporter activity for conjugated

compounds such as estradiol 17 β glucuronides [117], it is interesting to note that Sugiyama and colleagues [118] demonstrated a reduction in elimination of estradiol 17 β glucuronide from the brain of *Mrp1* knockout mice compared to that observed in the wild-type controls with functional MRP1. These functional studies support the luminal expression of MRP1 and suggest a role in limiting brain exposure to drugs and endogenous solutes.

6.6.2. MRP2

The expression and localization of MRP2 within the BBB is the subject of much debate. Studies by Miller et al. [119] indicated MRP2 was expressed in the luminal plasma membrane of isolated rat brain capillaries. These initial findings were supported by reports of MRP2 expression in both human brain capillaries as well as zebrafish [120,121]. In contrast, no detectable expression of MRP2 was found, at either the mRNA or protein level, in bovine brain microvessel endothelial cells [122,123] or mouse brain microvessel endothelial cells [110,118]. Furthermore, studies examining MRP2 protein expression in isolated human brain capillaries were below detection limits [114,124]. Interestingly, expression of MRP2 in rat brain endothelial cells was inducible by activation of either pregnane X receptor (PXR) or constitutive androstane receptor (CAR) pathways [125,126].

Functionally, MRP2 mediates the transport of glucuronide and GSH conjugates to a lesser extent than MRP1 [127]. It also actively transports chemotherapeutics such as methotrexate, vinca alkaloids, anthracyclins, antiepileptics such as phenytoin and endogenous agents like leukotriene C4 [107,109,128,129]. Thus if MRP2 is expressed in the BBB, it could have a profound effect on the brain distribution of many therapeutic agents. However, there are few studies showing a significant impact of MRP2 on the BBB permeability. One such study demonstrated an increased accumulation of phenytoin in the brain of *Mrp2* deficient rats compared to controls [129]. There is also evidence for MRP2-mediated changes in brain penetration of drugs in epileptic animals. Based on available information, most evidence indicates that MRP2 expression in the BBB is low or below detectable limits and as such has negligible effects on solute and macromolecule distribution into the brain. However, as MRP2 expression appears highly inducible, there is a possibility that MRP2 activity in the BBB could be of importance during pathological events within the CNS.

6.6.3. MRP3

Studies by Zhang et al. [122] identified low and variable expression of MRP3 in bovine brain microvessel endothelial cells. Subsequent proteomics based studies of both mouse [130] and human [131] BBB indicated that MRP3 expression was below detection limits.

6.6.4. MRP4

Evidence supporting a significant functional role for MRP4 in the BBB is perhaps the strongest of all the MRPs. The first evidence of MRP4 expression in the BBB was the studies by Zhang et al. [122] in bovine brain microvessel endothelial cells. Follow-up studies examining the localization of MRP4 suggested both luminal and abluminal presence of the

transporter [115]. The expression of MRP4 has since been reported in human, mouse and rat BBB [114,116,132]. Comparison of MRP4 expression in the brains of wild-type and *Mrp4* knockout mice confirmed BBB localization as well as expression in the choroid plexus epithelial cells forming the BCSFB [132].

Functionally, MRP4 can transport a wide variety of substrates and is important in the efflux of many nucleotide analog based chemotherapeutics. As with MRP1 and MRP2, MRP4 transports the endogenous substrate leukotriene C4 [133,134]. However, in addition, MRP4 can also transport endogenous nucleotides such as cAMP and cGMP [135]. Common chemotherapeutic purine nucleotide anion analogs that are effluxed by this transporter include bis(pivaloyloxymethyl)-9-[2-(phosphonomethoxy)ethyl]-adenine (PMEA), and active metabolites of 6-mercaptopurine and 6-thioguanine [72,107,136]. Using *Mrp4* $-/-$ knockout mice significant increases in topotecan [132] and PMEA [137] accumulation in the brain was observed.

6.6.5. MRP5 and MRP6

Within the BBB, MRP5 is highly expressed, whereas MRP6 is expressed to a lesser extent [122]. Presently their locations within the BBB remain unclear. Previous studies by Zhang et al. [115] found MRP5 protein expression to be primarily in the apical membrane fraction of brain microvessel endothelial cells. These findings were supported by Nies et al. [114]. In contrast, Roberts et al. [116] found low levels of abluminal MRP5 expression when staining in rat brain microvessel endothelial cells. Currently, the location of MRP6 remains to be seen because no specific MRP6 antibody is available at this time [115].

MRP5 can transport purine nucleotide analogs [127] and is the primary active transporter of cGMP and cAMP [138]. Therefore MRP5 and -4 may work in concert to regulate cGMP and cAMP levels [127] in the brain. MRP6 can transport anionic organic ions but cannot transport glucuronide or GSH [127] and has been shown to transport leukotriene C4 [139].

6.6.6. MRP7, -8 and -9

Currently, little is known about these transporters with regards to the BBB. MRP7, -8 and -9 have been found to be expressed in brain [140]. MRP7 can transport glucuronide E2 17betaG and exhibits high levels of resistance to taxane docetaxel, approximately 9 to 13 fold [140]. MRP8 is able to transport nucleotide analogues such as PMEA, glutathione conjugates and methotrexate [140]. No substrates for MRP9 have been identified at this time [109].

When looking at drug resistant efflux transport in the literature, major focus has been put on P-gp and BCRP and limited research has been focused on members within the MRP subfamily as it relates to the BBB. As has been demonstrated here, many of these MRP transporters can transport substrates that are important both physiologically and in the clinic. Particularly within the CNS, it is important to decipher any discrepancies in location and expression of MRP members within the BBB because they can have relevant impact on CNS drug concentrations reaching therapeutic levels within the brain and thus can affect our ability to treat important brain pathologies.

6.7. Breast Cancer Resistance Protein (BCRP)

BCRP was originally discovered in the MCF-7 AdrVp breast cancer cell line after observing that the cells are resistant to chemotherapeutic drugs including mitoxantrone, doxorubicin, and daunorubicin [141]. The gene sequence of the protein was isolated shortly after and was classified as the group G subfamily of ABC transporters. There are at least 5 members of ABCG subfamily identified in humans (ABCG1, ABCG2, ABCG4, ABCG5, and ABCG8). However, the primary form that plays a crucial role in the transport of substances between the blood and the CNS is ABCG2 [141]. At the protein level, ABCG2 is approximately 72-kDa with 665 amino acids and is considered as a half-transporter as shown in Figure 5. Other ABC transporters have two sets of membrane spanning regions (6 transmembrane α -helices) and two nucleotide binding domains (NBD); the G subfamily of protein consists of only one set of membrane spanning domain 6 transmembrane α -helices and only one NBD [141]. In order to function, it is believed that these half-transporters form homodimers [142].

The specific localization of ABCG2 within the CNS is primarily confined to the luminal plasma membrane of the brain microvessel endothelial cells. Given the localization within the BBB and the compounds that are transported by ABCG2, it has been suggested that ABCG2 most likely protects the brain from xenobiotics and toxins similar to other ABC transporters [141]. Furthermore, ABCG2 also plays a role in the accumulation and disposition of various endogenous substrates including sulfate and glucuronide conjugates of estrone and dehydroepiandrosterone [143,144]. In addition to endogenous substrates, ABCG2 also binds and recognizes a broad range of structurally-unrelated drugs and xenobiotics [141]. Many of these transport substrates also interact with other ABC transporters including ABCB1 and the ABCC subfamily; thus, the accumulation and distribution of drugs can be significantly altered.

6.8. P-glycoprotein (P-gp)

P-glycoprotein (P-gp) was the first ABC transporter to be characterized. First identified by Juliano and Ling [145] in 1976 using Chinese hamster ovary cells with selected resistance to colchicine, they discovered that the drug resistance properties of the mutated cells were consistently correlated with a high molecular weight component found in the plasma membrane with an approximate weight of 170,000 Da [145]. They also observed that the component was likely a glycoprotein associated with the plasma membrane of the mutated cells and was consistently absent or expressed at a lower level in the wild-type cells. Furthermore, they also noticed that the mutant cells with high levels of glycoprotein displayed an altered drug permeability; thus, they designated it as “P-glycoprotein” [145]. P-gp is also expressed in numerous tissues, including adrenal glands, kidneys, liver, colon, small intestine, heart, testes, peripheral nerves, and the brain. At the BBB, it is the most extensively studied ABC transporter being expressed in the luminal plasma membrane of brain endothelial cells [104]. Under normal conditions, the presence of P-gp in the BBB limits a broad range of substances from penetrating the brain tissue. Some notable drug classes with reduced brain penetration due to P-gp efflux at the BBB include anti-epileptics, anti-cancer drugs, anti-histamines and HIV protease inhibitors [103]. Numerous studies using drugs such as cyclosporine, digoxin, domperidone, etoposide, loperamide, ondansetron, taxol and vinblastine have shown the important role of

Figure 5. Structures of (A) MRP transporter, (B) BCRP transporter, and (C) P-glycoprotein transporter.

P-gp in the pharmacokinetics of P-gp substrates in multiple parts of the body. Table 1 shows some of the drugs that are known substrates for P-gp.

Similar to other ABC transporters, P-gp is a transmembrane protein with a molecular weight of 170 KDa formed by two homologous subunits that function as an efflux pump in an ATP-dependent manner (shown in Figure 5). The protein is assembled in two halves connected by

a 75 amino acid linker region. Each half contains 6 transmembrane segments, an intracellular nucleotide binding domain, and both intracellular N and C terminal regions. The exact localization of P-gp had been a subject of some debate with evidence supporting both luminal and abluminal expression of the protein. Luminal P-gp expression had been observed in rat and mouse brain capillaries [115,146,147]. Furthermore, the luminal expression of P-gp has also been isolated in human brain microvessels [113,148]. In contrast, electron microscope techniques have shown an enhanced expression of P-gp on the abluminal side of the rat brain endothelial cells [149]. Nevertheless, recent studies using immunoreactivity support the localization of P-gp on the luminal side of the endothelial cells [116].

Cancer Drugs	Immuno-suppressive Drugs	Lipid Lowering Agent	Steroids	HIV Protease Inhibitors	Cardiac Drugs	Anti-Diarrheal Drugs	Anti-Gout Agent	Anti-Bacterial Agents	Anti-Helminthic Agent
Doxorubicin	Cyclosporin A	Lovastatin	Aldosterone	Amprenavir	Digoxin	Loperamide	Colchicine	Erythromycin	Ivermectin
Daunorubicin	FK506		Cortisol	Indinavir	Quinidine	Antiemetics		Rifampin	Abamectin
Vinblastine	Tacrolimus		Corticosterone	Nelfinavir		Domperidone		Valinomycin	
Vincristine			Hydrocortisone	Ritonavir		Ondansetron		Gramicidin	
Vindesine			Dexamethasone	Saquinavir				Grepafloxacin	
Vinorelbine			Triamcinolone	Lopinavir					
Paclitaxel									
Etoposide									
Teniposide									
Epirubicin									
Irinotecan									
Tamoxifen									
Methotrexate									
Amsacrine									
Imatinib									

Table 1 Representative compounds that are known to be P-glycoprotein substrates. (Adapted from [156])

Numerous studies have attempted to identify and characterize P-gp substrates. Unlike conventional transporters, which recognize specific substrates, P-gp recognizes a broad range of compounds and has the capacity to extract its substrates directly from the plasma membrane [150]. Some of the most common features of P-gp substrates include their lipophilic nature that enables them to cross the lipid bilayer of the cell membrane. Furthermore, many P-gp substrates commonly consist of two aromatic rings and a basic nitrogen atom. These molecules can be uncharged or basic in nature, although some acidic compounds including methotrexate and phenytoin can also bind to P-gp but at a lower rate. Molecules with molecular weight ranges from 300 to 2000 Da are capable of binding to the protein and being transported [151]. Peptide substrates consisting of 3 to 15 amino acids with molecular weight ranges from 380 to

1880 Da can also interact with P-gp [151]. Most recently, beta amyloid protein, the component found in amyloid plaques in Alzheimer’s disease was reported to be a transport substrate of both P-gp but not BCRP [152,153].

One method for overcoming the limited permeability of P-gp transport substrates is to pharmacologically inhibit Pgp. P-glycoprotein inhibitors are themselves non-cytotoxic agents that can be used in combination with P-gp substrates to maintain the intracellular drug concentration. An inhibitor binds to P-gp and prevents the transport of P-gp substrates. There are at least three generations of P-gp inhibitors. The first generation compounds are less potent and non-selective with undesirable side effects at inhibitory concentrations. Examples of first generation inhibitors include the calcium channel blocker, verapamil, and the immunosuppressive agent cyclosporin A. First generation P-gp inhibitors act as competitive inhibitors of P-gp transport [154].

The second-generation compounds including dexverapamil or dextiguldipine were developed to reduce the toxicity associated with P-gp inhibition. They eliminate the undesirable side effects while retaining the ability to inhibit P-gp. The third generation inhibitors including tariquidar and elacridar are much more specific and more potent than earlier compounds. Unlike the first and second generation of P-gp inhibitors, the third generation of drugs acts as non-competitive inhibitors of P-gp, and the compounds themselves are not transported by P-gp [155]. Table 2 summarizes representative P-gp inhibitors [104].

Cyclopropylbenzosuberane	Immunosuppressant	Calcium channel blocker	Progesterone antagonist	Antiarrhythmic agent	Antifungal agent	Acridonecarboxamide derivative	Topoisomerase
LY335979	Cyclosporin A Valspodar (PSC833)	Verapamil	Mefiprostone (RU486)	Quinidine	Ketoconazole	GG918 (GF120918)	Xenova (XR 5944)

Table 2 Representative compounds that are known to be P-glycoprotein inhibitors. (Adapted from [156])

The ability of P-gp to extrude xenobiotics provides protection and detoxification of cells under normal conditions. For example, knockout mice (MDR1a^{-/-}) have been shown to be more sensitive to ivermectin and are susceptible to serious neurotoxicity compared to wild type control mice [156]. Considering the broad range of P-gp substrates and the expression of P-gp in tissues responsible for absorption, distribution and elimination of drugs, it is no surprise that this particular drug efflux transporter can significantly affect the absorption and distribution of drugs. This is especially true for cancer therapies used in the treatment of brain tumors. The tight junctions of the BBB restrict paracellular diffusion of chemotherapeutic agents into the CNS, while the presence of the various drug efflux transporters, such as P-gp, within the endothelial cells of the BBB reduces transcellular passage of chemotherapeutic agents into the brain and tumor sites.

7. Conclusion

The brain capillaries are structurally and functionally different from capillaries formed in the other organs. The selectiveness and permissiveness of the endothelial cell monolayer within the CNS is dependent on the tight junctions as well as the numerous transporter systems located on the luminal and the abluminal surface of the endothelial cells forming the BBB. The restrictive nature of the tight junctions along with transporter systems expressed in the BBB can significantly alter the accumulation and distribution of fatty acids and drugs in the CNS under pathological conditions. Improved delivery to the brain can be achieved by reversibly disrupting the physical tight junctions and/or inhibiting the activity of efflux transporter systems.

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