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REVIEW

Molecular structure and function of biological barriers

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ABSTRACT Biological barriers are indispensable for the integrity and function of many vertebrate organs. The barrier function is based on intercellular protein complexes of the plasma membrane which form paracellular diffusion barriers and separate internal and external fluid compartments, an indispensable prerequisite for every organ development and function. The review summarizes key characteristics and molecular structure of intercellular junctions (tight junctions and adherens junctions) responsible for cellular barrier formation. One of the most important such cellular barriers is the blood-brain barrier (BBB) which forms an active interface between the circulation and neural tissue. Its principal cellular components are cerebral endothelial cells, pericytes and astrocytes, whose finely tuned interactions are needed for a proper function. The review highlights the most important functions of the BBB including some novel regulatory aspects as well.
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Introduction

Parallel with the appearance of multicellular organisms, distribution of biological functions among different cells groups became a necessity. This distribution of functions – besides an increased evolutionary effectiveness – led to compartmentalization which became of vital importance for the proper function of higher multicellular organisms. The barrier between compartments is formed by specialized cell types. External surfaces of the body are covered by epithelial cells, while most internal surfaces are lined by endothelial cells, in the case of the blood-brain barrier these are specialised cerebral endothelial cells (CECs). These barriers control paracellular diffusion and are mainly formed by intercellular protein complexes of the plasma membrane. The most important structures sealing the paracellular route of transport (between the cells) are tight junctions (TJs), which are protein complexes joining together the membranes of adjacent cells. The backbone of the TJs is formed by claudin proteins.

The integrity and function of many vertebrate organs depend on these barriers, which separate internal and external fluid compartments, which is an indispensable prerequisite for the development and function of every organ.

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Most important organs with tight biological barriers

Skin

The epidermis is the outermost layer of the skin with direct contact to the environment and thus plays an important role in the protection of the organism. The epidermis is formed by several keratinocyte layers with different characteristics, organized in a multilayered, stratified epithelium. Tight junctions connect keratinocytes, and TJ proteins exhibit differential expression in different epidermal layers. Besides playing a pivotal role in barrier formation, TJ proteins of the skin may be involved in regulation of cell proliferation and differentiation and determination of tissue polarity. Several diseases including psoriasis vulgaris and atopic dermatitis are associated with alterations in TJ protein expression (Kirschner et al. 2012).

Kidney

In kidney the barrier is located along the renal tubule and separates urine from renal parenchyma. Different segments of the nephron have different paracellular transport characteristics, which is reflected by the distribution of junctional proteins in renal tubule epithelial cells as well. For example the pore-forming claudin-2 is expressed preferentially in the proximal tubule which has higher paracellular permeability, whereas

claudin-1 is more abundant in distal collecting segments (Kirk et al. 2010). Aberrant expression of claudin proteins could be implicated in a series of renal diseases (Balkovetz 2006) and could lead to abnormalities of reabsorption.

Intestine

The intestinal barrier, located at the level of epithelial cells covering the lumen of the intestine, separates the content of the intestine from the rest of the body. The integrity of the barrier is crucial not only for the absorption of nutrients but for host defence against pathogens and ingested toxic substances as well. Expression of junctional proteins in intestinal epithelial cells is under the control of a complex regulatory machinery. Expression of TJ proteins is altered not only in a large variety of intestinal diseases but also in colorectal cancer (Lu et al. 2013).

Liver

In the liver the barrier is localized along the bile canaliculi forming the blood-biliary barrier and bile ducts. This is reflected by the localization of TJ proteins as well, which are mainly expressed in the apical region of hepatocytes forming bile canaliculi and bile duct epithelial cells (cholangiocytes). The liver is very abundant in TJ proteins which play pivotal role in the regulation of paracellular permeability, bile secretion, and cell polarity. Beside this, TJ molecules may be involved in mediating Hepatitis C virus infection and it has been suggested that liver cancer is also associated with changed TJ protein expression (Lee and Luk 2010).

Lung

In the lung an active barrier is located at the interface of blood and airspace. The principal cellular elements of this barrier are lung epithelial cells which express at least 14 different types of claudins, of which claudin-3, claudin-4, and claudin-18 are the most prominent. Deterioration of the barrier function, due to altered TJ protein expression associated with several diseases, can lead to pulmonary oedema formation (Koval 2013).

Further organs in which functional biological barriers play an important role include *the testis and the brain.* In the testis the barrier is located between adjacent Sertoli cells of the seminiferous tubule, forming the blood-testis barrier. The central nervous system (CNS) is protected from the external environment by the blood-brain barrier (BBB) located to endothelial cells of cerebral capillaries and the blood-CSF barrier located to the epithelial cells of the choroid plexus. The blood-brain barrier will be discussed in more detail below.

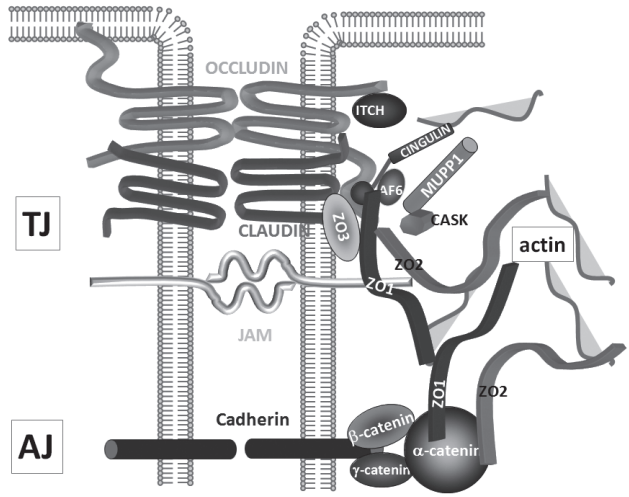

Figure 1. Molecular structure of the interendothelial junctions. Schematic representation of interendothelial junctional complexes presenting the transmembrane and cytoplasmic plaque components of the TJ and AJ.

Molecular basis of the cellular barrier formation

Barrier forming cells are interconnected by multiple types of intercellular junctions including TJs, adherens junctions (AJs), desmosomes and gap junctions. However, in the barrier formation TJs and AJs play by far the most important role (Fig. 1).

Tight junctions

Tight junctions are cellular structures located to the region where two cells virtually join their membranes together forming an impermeable barrier between the cells. TJs are composed of a branching network of TJ strands whose complexity is in direct relation with the impermeability of the barrier. TJs have a double function. By sealing the intercellular cleft they restrict the free movement of different solutes between the adjacent cells (barrier function). In addition, by restricting the free movement of membrane proteins TJs separate the apical membrane from the basolateral membrane, thus contributing substantially to the development of cell polarity (fence function).

Tight junction proteins

Most of our knowledge regarding tight junction structure and function originates from studies performed in epithelial cells. Although there are some differences between endothelial and epithelial cells in this respect, the assembly principles remain

similar. Proteins of the TJs fall into two categories: transmembrane proteins and cytoplasmic plaque proteins.

Transmembrane proteins of tight junctions

Although tight junctions have been described earlier, identification of the sealing elements of the junctional cleft started with the discovery of the first transmembrane protein of the tight junction by Furuse et al., which was occludin (Furuse et al. 1993). Since then the number of identified TJ transmembrane proteins increased rapidly and two main classes can be identified.

Tetraspan proteins

Tetraspan proteins, characterized by four transmembrane domains include claudins and the Marvel family of proteins.

After the initial discovery of claudin-1 and -2 (Furuse et al. 1998) now we know at least 27 members of the claudin family (Mineta et al. 2011). Claudins are relatively small proteins (20-27 kDa) and constitute the backbone of the tight junctions. In CECs claudin-5 is by far the most abundant family member, but claudin-1, -3 and -12 are also present in relevant amounts. The importance of claudin-5 is demonstrated by knock-out animals: claudin-5 loss opens the BBB for molecules smaller than 800 Da (Nitta et al. 2003). Depending on the composition of the extracellular loops, claudins have distinct functions: some of them are tightening the paracellular barrier, while others are pore-forming. Claudins are capable of both homo- and heterophylic interactions and can bind other claudins in trans interactions between cells and in cis within the membrane of the same cell (Krause et al. 2008; Van Itallie 2013).

The Marvel family of transmembrane TJ proteins consists of occludin, tricellulin (Marvel D2) and Marvel D3. The membrane topology of occludin – which has several alternative splice variants – is very similar to that of claudins; however, they do not show sequence homology, and occludin is significantly larger compared to claudins (60-65 kDa). Interestingly, occludin is not indispensable for TJ formation as revealed by studies performed on occludin-deficient mice (Saitou et al. 2000). Instead occludin may have a regulatory and accessory function in TJ formation and physiology. Occludin can be regulated by posttranslational modifications, especially phosphorylation on serine, threonine (Cordenonsi et al. 1999) or tyrosine (Tsukamoto and Nigam 1999; Chen at al. 2002) residues; can be internalized and is subject proteolytic degradation (by metalloproteinases or the proteasome) (Traweger et al. 2002).

Tricellulin was first described in epithelial cells as a protein localized to tricellular contacts. Later tricellulin has been detected in bicellular contacts and recently it has been detected in cerebral endothelial cells as well (Mariano et al. 2013).

MarvelD3 has also been shown to be expressed in both epithelial and endothelial cells (Steed et al. 2009), and it was suggested that occludin, tricellulin and MarvelD3 have overlapping but non-redundant functions (Raleigh et al. 2010).

Single span proteins

Single span TJ molecules belong either to the immunoglobulin-like superfamily or are non-immunoglobulin-like single span proteins. The most prominent members of the immunoglobulin-like superfamily single span molecules are the junctional adhesion molecules (JAMs) characterized by two extracellular loops and a single transmembrane domain. JAMs typically form homotypic cell-cell contacts between endothelial or epithelial cells. Endothelial cells express JAM-A, JAM-B and JAM-C and they play a role in the transendothelial migration of leukocytes (Jia et al. 2013). Further Ig-superfamily protein members shown to be localized to TJs include CAR (coxsackie- and adenovirus receptor) (Cohen et al. 2001), CLMP (coxsackie - and adenovirus receptor-like membrane protein) (Raschpberger et al. 2004), JAM-4 (Hirabayashi et al. 2003), and ESAM (endothelial cell-selective adhesion molecule) (Hirata et al. 2001); however, their role in the formation of the BBB is largely unknown. More recently three new Ig-like proteins, the angulins were found to be localized into the tricellular junctions and are required for the assembly of this structure (Higashi et al. 2013). The present knowledge about the role of non-immunoglobulin-like single span molecules like CRB3 or BVES/Pop1 (blood vessel/epicardial substance) in the formation or maintenance of the BBB is scarce. CRB3 regulates epithelial development (Whiteman et al. 2014) and BVES/Pop1 co-localizes with ZO-1 and occludin in polarized epithelial cells.

Plaque proteins of the TJs

The connection between transmembrane proteins of tight junctions and the cellular cytoskeleton is mediated by peripheral (cytoplasmic or plaque) tight junction proteins. These include proteins containing PDZ domains, of which MAGUK (membrane-associated guanylate kinase) proteins are the best characterized so far. The most prominent subgroup is represented by the zonula occludens (ZO) proteins having a modular nature consisting of PDZ domains, an SH3 domain, followed by a catalytically inactive guanylate kinase (GUK) domain. ZO-1 and ZO-2 are present in both epithelial and endothelial cells, whereas the expression of ZO-3 seems to be restricted to epithelial cells. Non-PDZ proteins localized to the TJ are cingulin and JACOP (junction-associated coiled-coil protein)/paracingulin; their role in the function of BBB

has not been characterized. Importantly, some of the peripheral TJ proteins serve not only as molecular scaffolds, but can function as cellular signalling molecules as well (Farkas et al. 2012; Traweger et al. 2013). In recent years a considerable number of other junctional proteins have been described, but their role still needs to be clarified. In addition, several other regulatory proteins have been demonstrated to localize to the TJ-plaque, these include protein kinases (Chen et al. 2002), heterotrimeric G-proteins (Saha et al. 1998) cyclin D1 (Capaldo at al. 2011) and Rho family GTPases (Quiros and Nusrat 2014). These findings indicate that the TJ is a dynamic structure able to respond to environmental stimuli and is regulated by a sophisticated signalling network responsible for the fine tuning of paracellular permeability.

Adherens junctions

Adherens junctions are present in many cell types and are located just below the TJ complex. The intimate connection to the TJs is indicated by the existence of common structural elements, like ZO-2 (Itoh et al. 1999). The transmembrane proteins of the AJs are the classical cadherins (N, -E, -VE, -P cadherins) which are single span transmembrane proteins capable of homophylic Ca^{2+}-dependent binding. The most prominent cadherin of cerebral endothelial cells is VE-cadherin, but the presence of N-cadherin has been documented as well (Dejana and Orsenigo 2013). Cadherins are linked to the actin cytoskeleton through catenins (α-, β-, γ- and p120-catenin), of which β- and γ-catenin can bind directly to cadherins. In addition, β-catenin participates directly in signal transduction as well.

Another major protein complex of adherens junctions consists of nectins linked to afadin/AF6. PECAM-1 also participates in the formation of endothelial adherens junctions. Adherens junctions are very important not only in cell adhesion, but in cell migration and thus metastasis formation as well.

Phosphorylation of junctional proteins

Practically all components of the TJ and AJ are phosphoproteins and their phosphorylation status is crucial for regulating intercellular junction structure and function, and thus barrier properties. Occludin is phosphorylated by PKCζ and PKCη on specific C terminal threonines promoting TJ assembly (Jain et al. 2011), while S408 phosphorylation by casein kinase 2 (CK2) reduces the association of occludin with other TJ components (Suzuki et al. 2009; Raleigh et al. 2011). Interestingly, S490 phosphorylation of occludin is required for centrosome separation and mitosis (Runkle et al. 2011). The phosphorylation state of tricellulin also appears to closely associate with TJ function (Takasawa et al. 2013). Several members of the claudin protein family also can be phosphorylated. For example the phosphorylation of claudin-2 on S208 at the C terminus of the protein promotes its membrane retention. The cAMP-dependent PKA-mediated dephosphorylation of the same residue targets claudin-2 to lysosomes (Van Itallie et al. 2012). On the contrary, the corresponding serine of claudin-16 (S217) is phosphorylated in a cAMP/PKA-dependent manner (Ikari et al. 2008). Claudin-3 is also directly phosphorylated by PKA at T192 and this decreases its incorporation into TJs (D'Souza et al. 2005). On the other hand claudin-5 is increased at cell-cell junctions when it is phosphorylated in response to cAMP (Ishizaki et al. 2003). Phosphorylation of claudin-1 and -4 by aPKC is again necessary for TJ formation (Aono and Hirai 2008; Banan et al. 2005). aPKC can also directly phosphorylate the single span immunoglobulin like TJ protein JAM-A at S285 and promotes TJ maturation (Iden et al. 2012), while CAR is phosphorylated by PKCδ and this modification is important for TJ stability (Morton et al. 2013). The recently discovered single pass transmembrane protein of the tricellular TJ, LSR requires phosphorylation by JNK1/2 for proper localization to tricellular junctions (Nakatsu et al. 2014).

We used phenylarsine oxide (PAO) an inhibitor of protein-tyrosine phosphatases (PTPs) to study the effect of tyrosine phosphorylation on the blood-brain barrier. As shown in Fig. 2A, inhibition of PTPs induces a drop of transendothelial electric resistance (TEER) as early as 20 minutes after the start of treatment. Furthermore, we analysed the distribution of selected junctional proteins in Triton x-100 soluble and insoluble fractions to detect shifts in the more membrane associated and more cytoskeleton associated protein pools in response to PAO treatment. As presented in Fig. 2B, occludin and ZO-1 levels increase in the Triton X-100 soluble fraction in response to 1 hour 30 μM PAO treatment, while the same proteins exhibit a drastic decrease in the Triton X-100 insoluble pool. Inversely, PAO treatment results in a drop of ZO-2 and β-catenin levels in the soluble pool while both are greatly increased in the insoluble pool.

Thus it is clear that differential phosphorylations of junctional components are principal regulators of barrier homeostasis.

The blood-brain barrier (BBB)

The BBB is an active interface with an estimated surface of about 10-20 m^2, which separates the systemic circulation from the CNS, and strictly controls the molecular and cellular traffic between the blood and the brain. Its proper function is indispensable for the maintenance of a steady-state environment needed for neuronal function.

It was Paul Ehrlich who in 1885 (Ehrlich 1885) observed that some dyes injected intravenously stained strongly various organs, but the brain remained largely unstained. This observation led to investigations (Lewandowski 1900; Gold-

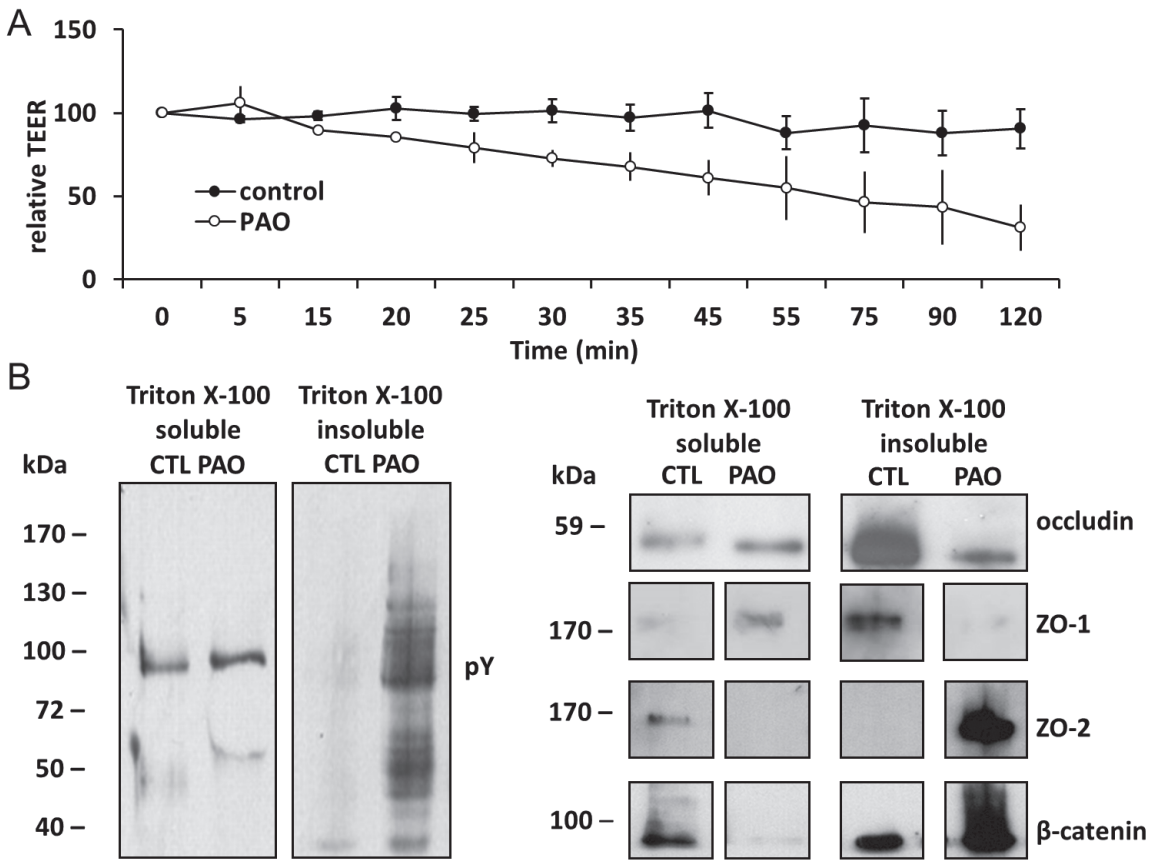

Figure 2. Inhibition of tyrosine phosphatases affects junctional protein distribution. Cells were treated with protein tyrosine phosphatase inhibitor phelylarsine oxide (PAO) at 30 μM. **A** TEER decreased by over 50% within two hours. **B** Marked tyrosine phosphorylation was observed over a wide molecular weight range in the Triton X-100 insoluble fraction of cell lysates by Western blot. The increased tyrosine phosphorylation resulted in a redistribution of junctional proteins between the Triton X-100 soluble and insoluble fractions.

mann 1913) to identify what blocks the entry of hydrophilic molecules from the blood to the brain. The term blood-brain barrier was created by Stern and Rothlin (1918) nearly 100 years ago. Using electron microscopy and molecular tracers, Reese and Karnowski (1967) clearly demonstrated that the barrier is located at the level of cerebral endothelial cells. In 1987 Janzer and Raff described the essential modulatory role of astrocytes and the 1990s witnessed the era of molecular dissection of the tight junctions, the cellular structure crucial for the formation of the paracellular barrier.

Acknowledging the special role of the cerebral endothelium, the importance of other cellular components like astrocytes and pericytes in the formation of the BBB has become increasingly evident, which led to the formation of the concept of "neurovascular unit" (Neuwelt 2004).

Cellular composition of the BBB

The principal cellular components of the BBB are cerebral endothelial cells (CECs), pericytes and astrocytes (Fig. 3). Microglia and nerve endings as may have regulatory roles as well.

Endothelial cells

Cerebral endothelial cells are elongated cells of mesodermal origin lining the inside of cerebral capillaries. They constitute around 0.1-1% of the brain volume; however, they are involved in the pathogenesis of a significant number of CNS diseases. CECs have the common characteristics of endothelial cells, including expression of factor VIII-related antigen, high alkaline phosphatase and γ-glutamyl transpeptidase activity, specific lectin binding (*Bandeira simplicifolia* lectin B4) and uptake of acetylated-LDL. In addition, these cells have characteristics specific to epithelial cells as well, which confer them the barrier characteristics: presence of continuous strands of tight junctions sealing the intercellular cleft between adjacent CECs, very low pinocytotic and transcytotic

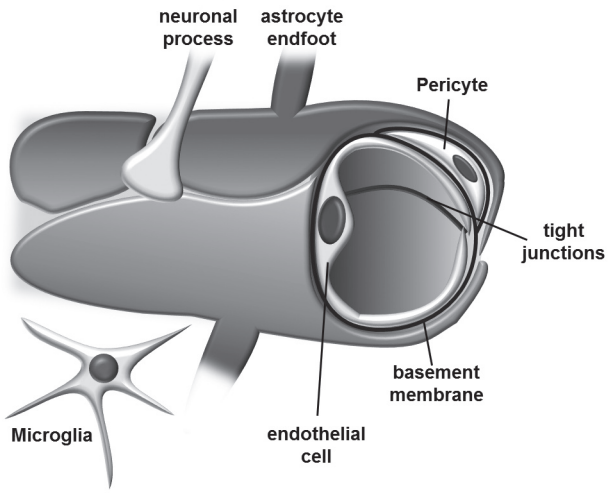

Figure 3. Cellular structure of the neurovascular unit. Graphic representation of the neurovascular unit showing the spatial relations of its cellular components. Microcapillaries are lined by endothelial cells which in turn are sheathed in the basement membrane. Pericytes are closely associated with the endothelial cells as they reside within the basement membrane. The endfeet of astrocytes cover close to the entire outer surface of the capillary leaving only small patches for association with neural processes.

activity, presence of specific enzymes, low level of adhesion molecules, expression of specific transporter systems and presence of a high number of mitochondria suggesting a high energy requirement of these cells. Besides common characteristics, cerebral endothelial cells show a remarkable heterogeneity (Krizbai et al. 2000) and responsiveness to extracellular stimuli modulated by a sophisticated intracellular signalling network (Fabian et al. 1998; Wilhelm et al. 2007; Hutamekalin et al. 2008).

Astrocytes

Astrocytes are cells of neuroectodermal origin with a large variety of morphology depending on their location and activation state. They do not play significant role in in the formation of the physical barrier; however, their bidirectional interaction with CECs is important in the maintenance of the functional integrity of the BBB. Astrocytes contact the capillaries by specialized structures called endfeet, which nearly completely ensheath endothelial cells and pericytes of the capillaries. In post-capillary venules the contact is not so intimate, which allows the formation of a perivascular space, and in arterioles a smooth muscle layer separates endothelial cells from astrocytes (Abbott et al. 2006). Astrocytic endfeet are rich in mitochondria, caveolae, coated pits and vesicles, indicating an active material exchange and high energy demand. Astrocyte endfeet express high levels of aquaporin-4 (forming the OAPs – orthogonal array of intramembraneous particles), connexin-43, the purinoreceptor P2Y4 and the Kir 4.1 K^+ channel.

Molecular mechanisms of the interaction between astrocytes and endothelial cells are complex (Fig. 4), and our knowledge in this respect is far from being complete. Besides a physical contact, astrocytes synthesize biologically active molecules which modulate the BBB phenotype. Such factors are transforming growth factor-β (TGF-β), glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), IL-6 (Haseloff et al. 2005) or Sonic hedgehog (Alvarez et al. 2011). Recent proteomic analysis revealed changes in 55 proteins in CECs in response to astrocytes,

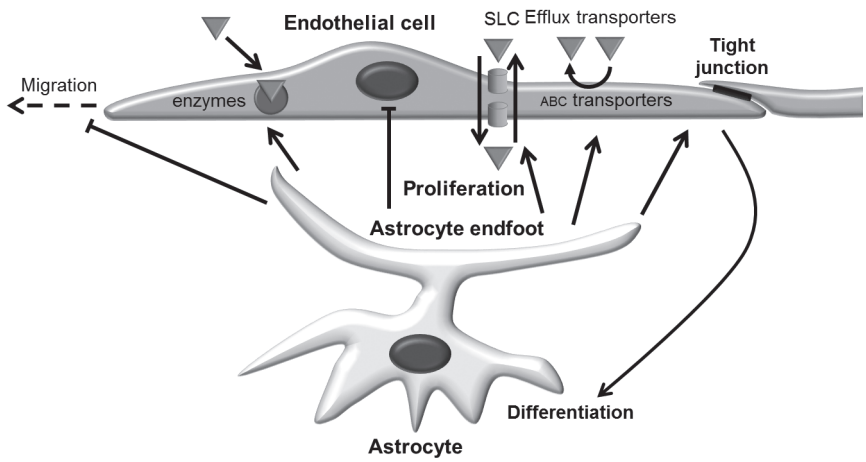

Figure 4. Cerebral endothelial cell-astrocyte interactions. Cartoon depicting the close association of astrocytes with endothelial cells. Even though the astrocytes are not physically part of the barrier, they are in intimate, bidirectional regulatory relation with endothelial cells.

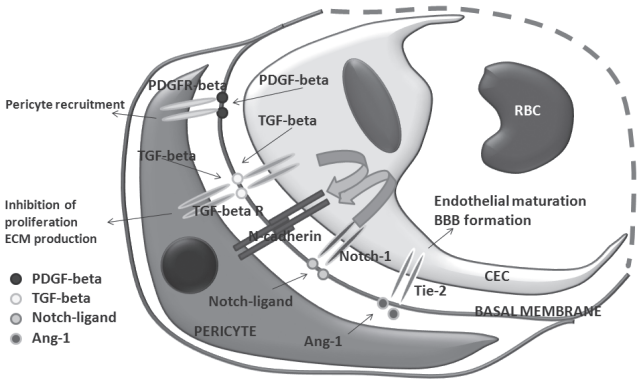

Figure 5. Cerebral endothelial cell-pericyte interactions. The interaction between pericytes and endothelial cells depicted in this figure are very complex. For example endothelial cells regulate pericyte recruitment, and in turn pericytes are important for endothelial maturation. Both cell types affect the other with regards to proliferation, migration and attachment through N-cadherin via TGF-β and Notch signalling.

involved mainly in cell structure and motility and protein metabolism and modification (Pottiez et al. 2011). In addition, extracellular matrix produced by astrocytes can induce BBB characteristics in brain endothelial cells.

On the other hand, endothelial cells also influence astrocytes. As such, endothelial cells increase the number of OAPs in the astrocytic endfeet, can induce the expression of plasminogen activator inhibitor-1 mRNA in astrocytes and CECs-derived LIF has been shown to induce astrocytic differentiation (Mi et al. 2001).

Pericytes

Pericytes were described in 1873 by the French scientist Charles-Marie Benjamin Rouget and were originally called Rouget cells. Later they were renamed to pericytes, due to their anatomical location abluminal to the endothelial cell and luminal to parenchymal cells. Pericytes are a very heterogeneous cell population, and the only characteristic which identifies them is their location. Several markers have been used for their identification, including smooth muscle actin, PDFGR-β, chondroitin sulfate proteoglycan 4 (CSPG4/NG2), desmin, but none of them is specific only to pericytes and is expressed in all pericytes.

Pericytes are localized in the duplication of the basement membrane covering approximately 22-32% of the endothelial surface. The ratio of pericytes to CECs varies from species to species: in the rat capillary is 1:5, in the mouse the ratio is 1:4 and in humans 1:3-1:4. Pericytes function at the BBB in at least two ways: by regulating BBB-specific gene expression patterns in endothelial cells, and by inducing polarization of astrocyte endfeet surrounding CNS blood vessels. The interaction between pericytes and endothelial cells is very complex (Fig. 5). Endothelial cells secrete PDGF-β, which plays a crucial role in pericyte recruitment to capillaries. An important molecule in endothelial cell-pericyte communication is TGF-β, secreted by both pericytes and CECs, and both cells express receptors for TGF-β. TGF-β is able to inhibit pericyte proliferation, but also enhances pericyte attachment to CECs mediated by the upregulation of N-cadherin. In endothelial cells, depending on which receptor type is activated, TGF-β can upregulate proliferation and migration of CECs or induce differentiation, inhibition of proliferation, and stabilization of the BBB. Ang-1 expressed by pericytes is necessary for vessel stability and maturation, a process mediated by Tie-2 receptors. An important role in the communication between CECs and pericytes plays Notch signalling which is able to regulate pericyte attachment to endothelial cells via N-cadherin (Hill et al. 2014). In addition, it seems that the differentiation stage of pericytes determines their effect on the endothelium as well (Thanabalasundaram et al. 2011).

The importance of pericytes in the formation of the BBB is supported by the finding that loss of pericytes by targeting the PDGF-β signalling pathway, leads to endothelial hyperplasia, abnormal vasculogenesis (Hellstom et al. 2001) and an increased BBB permeability, which is mediated mainly by increased endothelial transcytosis (Armulik et al. 2011).

The basement membrane

The basement membrane covers endothelial cells and astrocyte endfeet and engulfs pericytes. All three cell types contribute to its formation. It is composed of collagen (especially collagen type IV), elastin, laminin, fibronectin and proteoglycans (heparan sulfate and glycosaminoglycans). In addition, adhesion and signalling molecules may also be present. Components of the basement membrane may regulate barrier properties of the endothelial layer (Hartmann et al. 2007), and alterations in the basement membrane has been associated with increased BBB permeability observed under pathological conditions (Cardoso et al. 2010).

Role of the BBB

Due to its location between the neural tissue and systemic circulation, the blood-brain barrier regulates the transport of different molecules and cellular elements between the two compartments. These functions imply that the BBB has a considerable contribution to the maintenance of the homeostasis of the CNS required for proper neural function. The BBB has a dual function, a barrier and a carrier one.

Barrier functions

The barrier function consists of restricting the transport of potentially toxic or harmful substances and cells from the

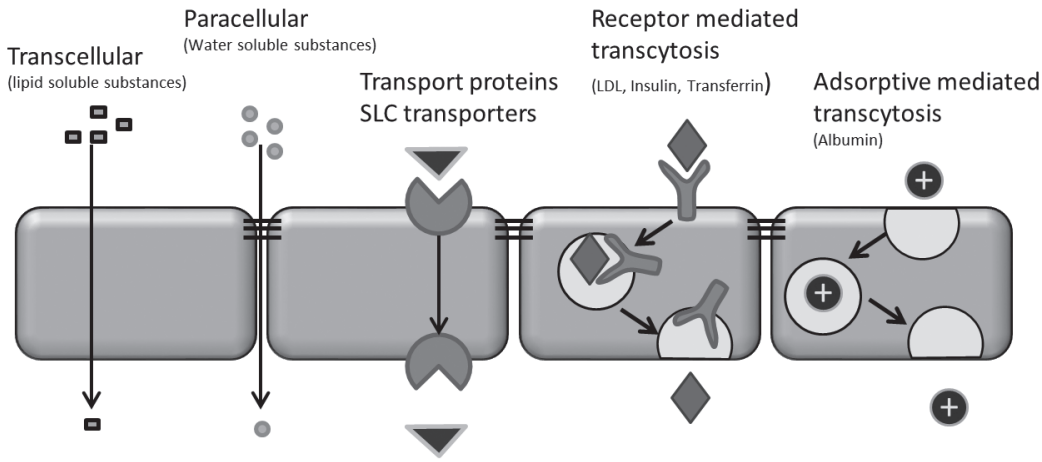

Figure 6. Transport pathways through the BBB. Schematic representations of transport routes though the BBB. The two main groups are paracellular and transcellular transport. The paracellular transport is regulated by the protein composition of the TJ. Transcellular transport can occur via diffusion in case of lipid soluble molecules or can be mediated by transporter proteins, receptors or adsorption.

blood to the brain. For this the BBB is equipped with a four-fold defence line. The paracellular barrier has its cellular and molecular basis in the closely apposed cerebral endothelial cells interconnected by a continuous line of tight junctions, which makes passage between the cells practically impossible. The transcellular barrier is provided by the low level of endocytosis and transcytosis, which characterizes cerebral endothelial cells under physiological conditions. The cerebral endothelium is equipped with a whole set of enzymes which form an enzymatic barrier. Endothelial enzymes which are capable of degrading biologically active substances entering the cells include acetylcholinesterase, alkaline phosphatase, γ-glutamyl transpeptidase and monoamine oxidases. The fourth defence line is provided by efflux transporters or ABC (ATP-binding cassette) transporters. These transporters use the energy of ATP to transport – against the concentration gradient – a wide variety of lipophilic substrates which can freely pass cellular membranes. Unfortunately, important drugs and drug candidates are also substrates of these transporters, which therefore constitute a major impediment in the therapy of CNS diseases. The most abundant ABC transporters of the cerebral endothelium are ABCB1 (P-gp, MDR – multidrug resistance protein), members of the ABCC family (MRPs – multidrug resistance-related proteins) including ABCC1, ABCC4, ABCC5, and ABCG2 (BCRP – breast cancer related protein). It is important to note that ABCG2 plays a more important role than ABCB1 in human capillaries in comparison to rodent brain capillaries.

Carrier functions

Some of the important nutrients of the brain are hydrophilic molecules, like glucose and amino acids, which are not able to freely pass through cellular membranes. Additionally, hydrophilic waste products of cell metabolism need to be removed from the brain. Therefore, the cerebral endothelium is equipped with a whole set of transporters which enable nutrient transport to the brain and removal of metabolites. These transporters belong to the solute-like carrier group of membrane transporters (SLC), having more than 50 families and over 300 transporters. These use facilitated transport (which allow solutes to flow downhill their electrochemical gradients) or secondary active transport (flow of solutes against their electrochemical gradient coupled to transport of a second solute that flows downhill with its gradient).

One of the most important SLC transporter of the BBB is the glucose transporter SLC2A1 (GLUT1), a 12 transmembrane domain containing protein with Km 2-5 mM for glucose. Further transporters include: members of the SLC3 and SLC7 family, transporting cationic and neutral amino acids (SLC7A5=LAT1, SLC7A6=LAT2, SLC7A3 system Bo+ – basic amino acid preferring); SLC15 (proton/oligopeptide transporters – PEPT1, 2); SLC16 (monocarboxylate transporters – MCT: lactate, pyruvate, hydroxybutyrate); SLC21(organic anion transporters – OATPs); SLC22 (organic anion, zwitterion, cation transporters) and SLC38 (sodium-coupled neutral amino acid transporters, including system A, alanine preferring SLC38A2, and system N, glutamine preferring SLC38A5).

In addition to these, cerebral endothelial cells are equipped with special transporters which transport biologically active molecules, like neurotransmitters. Glutamate transporters EAAT1 (SLC1A3) and EAAT2 (SLC1A2) have a special importance as they may participate in the removal of glutamate excess accumulated in response to brain ischemia. Besides this, it has been demonstrated that the cerebral endothelium

is able to transport serotonin (SLC6A4) (Brust et al. 2000) and histamine as well (SLC22A3). Since the tight junction is largely impermeable to ions under physiological conditions, transfer of ions is mediated by different ion transporters. These include Na^+/K^+ ATP-ase, $Na^+/K^+/2Cl^-$ transporter (SLC12A), Na^+/H^+ antiporter, H^+ ATP-ase and saturable Cl^- transporter. Principal transport routes across the BBB are shown in Figure. 6.

Receptors on cerebral endothelial cells

In order to adequately respond to environmental stimuli, CECs are equipped with a whole set of membrane receptors. These include adrenergic receptors, receptors for histamine (H1, H2), bradykinin, endothelin, opiate, glutamate, serotonin and adenosine. Furthermore, receptors involved in receptor mediated endocytosis are also expressed. The most important are: insulin receptor, transferrin receptor and LDL receptor. These receptors can be targeted for drug delivery to the brain.

Clinical aspects of the BBB

By controlling the traffic between the neural tissue and the systemic circulation, the BBB plays an important role in a large number of neurological disorders. Dysfunction of the BBB may be either a causative factor, or may be associated with a disease significantly affecting its outcome. The most significant BBB dysfunction associated with diseases is the increase in permeability, allowing potentially toxic substances to enter the brain and aggravate the damage. Pathological conditions with increased BBB permeability include stroke, brain trauma epilepsy, neurodegenerative disorders (Alzheimer's disease, ALS, Parkinson's disease), brain tumours (primary tumours and metastases), inflammatory disorders and CNS infections. The mechanisms of permeability change include increased paracellular permeability, increased pinocytosis, changes in endothelial cell surface charge or cytoskeletal changes. A special case of increased permeability is the increased cellular transmigration (immune cells, metastatic cells), which – under physiological conditions – is very restricted. Cellular transmigration can occur paracellularly or transcellularly as well (Carman and Springer 2004). The transmigration process starts with rolling of the cells and loose attachment to the endothelium, mediated by selectins, followed by firm adhesion, in which integrins play a crucial role. These steps are followed by the transmigration step (Engelhardt 2008).

The relative impermeability of the BBB may also cause severe clinical consequences, because the BBB constitutes the largest impediment for large or hydrophilic drugs to reach therapeutically relevant concentration in the brain. A low brain penetration has been observed for drugs which are substrates of efflux transporters as well. Therefore, one of the major challenges of drug industry is how to circumvent or breach the BBB specifically in order to target drugs to the brain.

Summary and conclusions

The principal cell types involved in barrier formation are epithelial and endothelial cells. Epithelial barriers interface the body and the external environment, such as in kidney, intestine, liver, lung, skin, brain choroid plexus; the main barrier based on endothelial cells is the BBB. The structural basis of paracellular barriers is formed by large protein complexes. Some constituent proteins have clearly characterised roles defining the structure and properties of the barrier, like the claudins. Other ostensibly structural proteins have been found to play crucial roles in modulating the barriers or even regulate gene expression and mitosis such as zonula occludens proteins and occludin. Regardless of their roles, all constituents of intercellular junctions are tightly regulated. One important attribute of the junctional proteins is their phosphorylation status which is finely tuned by the balanced action of kinases and phosphatases localized to the junctional complex.

A barrier with very special characteristics and functions is the BBB, located at the level of brain capillaries. Principal cellular components of the BBB are endothelial cells, astrocytes and pericytes. The BBB has a barrier function, which means that it restricts the transport from blood to the brain of potentially toxic or harmful substances. This is achieved by a fourfold defence line. Tight junctions form the paracellular barrier. Low levels of pinocytosis and transcytosis maintain the transcellular barrier. Unwanted peptides and small molecules are destroyed by the enzymatic barrier. Finally efflux pumps such as ABCB1, ABCC family members and ABCG2 act to remove toxins. The BBB also has a carrier function which implies transport of nutrients to the brain and removal of metabolites. In this process, SLC transporters play crucial role. Due to its strategic location between blood and CNS, the BBB plays an important role in the pathogenesis and treatment of a large number of neurological diseases.

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