The Blood-Brain Barrier

Victor Castro and Michal Toborek

Abstract The blood-brain barrier (BBB) is the anatomophysiological unit that interfaces between the blood and the brain. It is composed of brain vascular endothelial cells and their surrounding astrocytes and pericytes. These cells interface with neurons to form a functional unit that regulates blood flow in the brain and the traffic of substances between blood and brain parenchyma. The proper function of the BBB requires specialized roles for each of the cell types that compose it; thus, the endothelial cells form a proper biological barrier by expressing tight junctions (TJ) that seal the intercellular space while forming paracellular ion pores. The expression of TJ brings an additional benefit to the endothelial cells as they are determinants of membrane polarization; the resulting cell polarity is crucial for the proper expression of membrane transporters and ion channels responsible for the transcellular exchange of substances across the endothelium. The physiological properties of endothelial cells, however, are regulated by their interaction with astrocytes and pericytes that in turn interact with each other and nearby neurons. This chapter explores the cellular structure of the blood-brain barrier and provides an introduction to the molecular characteristics of tight junctions and electrophysiological properties of the brain vascular endothelium.

Keywords Blood-brain barrier • Microvasculature • Endothelium • Astrocytes • Pericytes • Transporting phenotype • Tight junctions

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1 Introduction

The blood-brain barrier (BBB) is a paramount determinant of brain homeostasis. It is a modulated anatomophysiological interface which separates and controls substance exchange between the blood and the brain parenchyma. Its discovery dates to the late nineteenth century, when Paul Ehrlich observed that if aniline-based dyes were injected into the vasculature of a living animal, most tissues would be readily stained, but not the brain or the spinal cord [1]. In 1898, Max H. Lewandowsky demonstrated that neurotoxins were able to affect brain functions if administered directly into the brain, but not if delivered through the vasculature [2]. Subsequently, in 1913, Edwin Goldman showed that the central nervous system (CNS) could be stained if the dyes were injected directly into the cerebrospinal fluid instead of systemic circulation; however, this staining was restricted to the brain, and not found in other organs [3], suggesting that the aniline compounds were not admitted into the circulating blood flow. This showed the existence of compartmentalization between the brain and the blood, and since no obvious separating membrane was found, it was suggested that the barrier resided directly in the bran microvessels. Later, with the introduction of electron microscopy, the barrier function was correlated to endothelial cells of brain capillaries.

2 Brain Vascular Endothelium

Epithelia are biological barriers that separate and maintain the physicochemical homeostasis between two biological compartments. They constitute a membrane formed by one or more layers of epithelial cells that adhere and communicate with each other, interact with the extracellular matrix, and rest on a basal lamina of connective tissue. To maintain homeostasis, epithelia are subspecialized to fulfill specific needs depending on their topological localization. Nevertheless, two features are common to all epithelial tissues regardless of their specialization: they are capable of vectorial transepithelial transport and they regulate the intercellular ionic flux (Fig. 1).

Endothelia are a subset of epithelia which form the interior lining of the heart, blood and lymph vessels, capillaries, and the serous cavities of the body. Endothelial cells are distinguished from epithelial cells by having different protein expression and molecular behavior patterns. However, similar to epithelia, endothelial cells are polarized, specialize in molecular transport, and regulate the intercellular flux of substances. In the circulatory system, the vascular endothelium ensures laminar blood flow, interacts with blood cells and plasma molecules, secretes hormones, expresses surface immunological recognition molecules, prevents thrombosis, and modulates the passage of immunological cells, water, and substances between the blood and the underlying tissues. In specific tissues (e.g., hepatic sinusoids and the

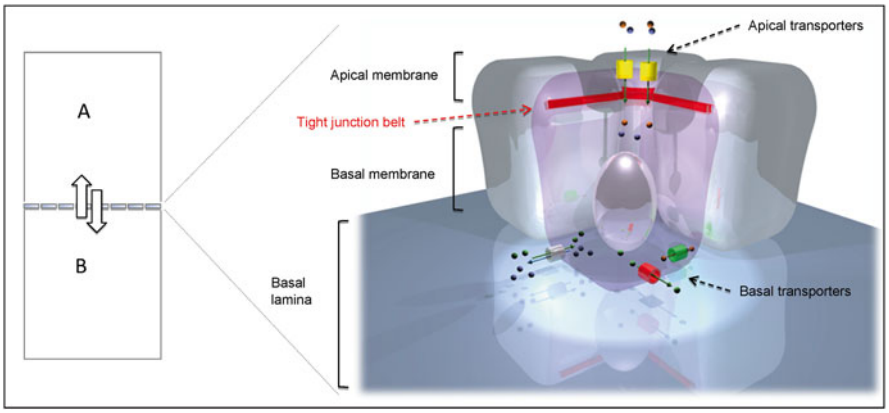

Fig. 1 Epithelia and endothelia are biological membranes that separate two biological compartments and are responsible for maintaining the physicochemical homeostasis between them. These two compartments are generally known as apical (A) and basal (B). The membrane of epithelial/endothelial cells is divided accordingly into apical and basal domains, separated by a molecular belt formed by tight junctions. Different sets of membrane receptors and transporters are expressed in each of these domains. As a result, the cell membrane is physically and functionally polarized

choroid plexus), the vascular endothelium is paired with an underlying epithelial membrane to form a dual transporting unit.

The vascular endothelium has paramount physiopathogenic roles in the onset and perpetuation of atherosclerosis and hypertension, and is involved in systemic inflammation processes, edema, and thrombosis. Furthermore, systemic viral and bacterial infections, and most therapeutic and recreational drugs disseminate through the blood, posing a challenge to the normal vascular endothelial physiology. Endothelial dysfunction is also involved in the progression of systemic metabolic diseases like diabetes mellitus or hypercholesterolemia.

The brain capillaries represent a special case of endothelial specialization and adaptation. Given the particular physicochemical isolation required by the brain, the capillary endothelial function must be regulated with precision, and proper mechanisms must be in place to ensure that brain functions are maintained during vascular growth and/or remodeling. To achieve this degree of regulation, the brain capillary endothelial cells rest on a basal lamina and form a cellular network with the surrounding pericytes and astrocytes that, in turn, are in contact with neurons. This cellular arrangement forms a neurovascular unit and constitutes the BBB, which is embedded in a thin layer of extracellular matrix and strictly regulates the transport of water, ions, glucose, nutrients, and other molecules to and from the brain, playing a major role in the removal of drugs and toxins from the brain parenchyma (Fig. 2).

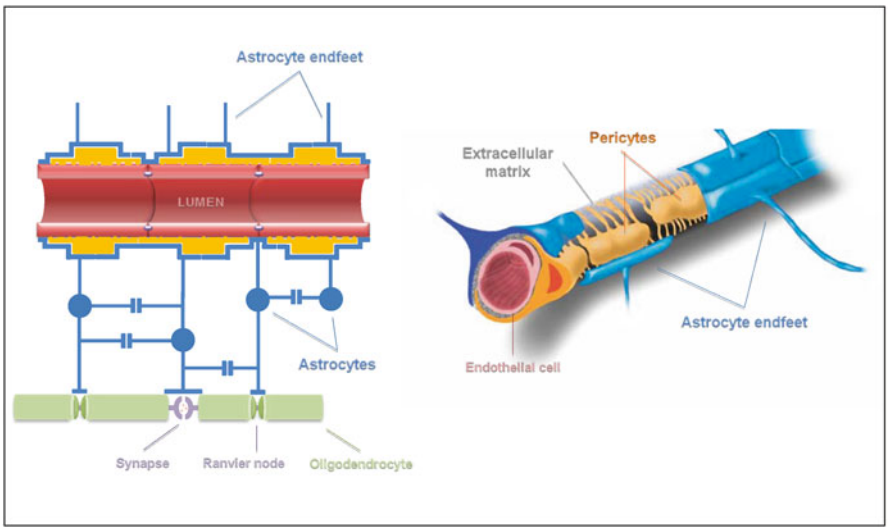

Fig. 2 The blood-brain barrier (BBB) is the regulated anatomophysiological interface that controls homeostasis between the blood and the brain parenchyma at the capillary level. Here, the brain vascular endothelium (*pink*) is surrounded by pericytes (*yellow*) and the end-feet of astrocytes (*blue*). They form a functional unit that is regulated by blood flow and neuronal activity. Astrocytes and pericytes play an important role in this regulation. Pericytes are in direct contact with endothelial cells, embracing them with multiple podocytes, to regulate blood flow and transduce signals to and from astrocytes and endothelial cells. Astrocytes form an extensive and well-organized network that interconnects capillaries with neurons and synapses. Specialized astrocyte end-feet are thus in direct contact with the synaptic space, Ranvier nodes, and brain capillaries

2.1 Transporting Phenotype

Electrophysiology experiments performed in the middle of the nineteenth century showed that electrical current measures in semi-intact frog-leg muscle preparations were consistently lower when the skin was conserved as compared to skin-free preparations [4]. These were the first published experiments that hinted the possibility of the skin modulating the electrical properties of the underlying tissues and prompted the first formal epithelial electrophysiological studies. Shortly after, it was discovered that the frog skin exhibited spontaneous electrical activity if placed between two compartments filled with ionic solutions, a condition that invariably resulted in the solution bathing the inner side of the skin (basal) becoming more electropositive than the solution bathing the outer side (apical) [5]. It was then shown that such spontaneous potential depended on the sodium and lithium ions present in the solutions used, so it was proposed that the electric activity of the frog skin resulted from the asymmetric epithelial permeability to those ions [6, 7].

Further studies demonstrated unambiguously that the skin is indeed capable of transporting Na^+ and that this transport occurs preferentially from the outer side to the inner side of the skin regardless of electrochemical gradient.

Based on these observations, epithelia and endothelia became represented as an equivalent electrical circuit that was originally represented as a "two-membrane" model, where the net transepithelial transport was equal to the net flux of Na^+ across the outer and the inner cell membranes [8]. To explain this phenomenon, it was proposed that the luminal membrane allowed Na^+ to passively enter the cell while the basal membrane transported it out while being exchanged by K^+. Thus, the model required a mechanism to pump Na^+ ions out of the cell through the basal membrane against their electrical and concentration gradients [9]. Incidentally, in a series of unrelated experiments, a ubiquitous membranal protein that actively transported Na^+ ions against their concentration gradient was described [10]. This protein, later identified as the Na^+/K^+-ATPase, was proposed to be the Na^+ pump required in the model. A direct functional implication was that two different sets of membrane transporters had to be expressed differently in the apical and basal regions of the cell membrane in order to explain the different transporting capabilities of both regions.

Further studies demonstrated that in the absence of paracellular free diffusion of ions (electric paracellular leakage), the apical and basal membranes of epithelial and endothelial cells had independent electrical properties and their combined voltage could be added, as if they were in a serial circuit. Thus, the intercellular space had to be closed to prevent the transported ions from freely diffusing back along their concentration gradients.

Biological barriers transport not only ions but also water and other solutes (e.g., glucose and amino acids) that are needed to maintain the homeostasis between the two biological compartments they separate. Endothelial transport occurs by transcellular and/or paracellular routes. Transcellular transport is determined by the presence or absence of specific membrane transporters and channels, whereas the paracellular route is regulated by tight junctions (TJs), protein complexes sealing the paracellular space and forming paracellular ion channels.

TJs are expressed as a continuous belt around the lateral borders of cells, dividing the cell membrane into apical and basal domains, preventing free diffusion of membrane receptors, transporters, and ion channels from one domain to the other, Effectively polarizing the cells. The vectorial transport of Na^+ from the apical to the basal side of the epithelium/endothelium generates a transepithelial/endothelial electrochemical gradient that constitutes the driving force for all other membrane transporters to work [11]. Thus, the transcellular exchange of glucose, amino acids, water, ions, toxins, drugs, etc. is driven by the transport of Na^+, whose vectoriality is determined by the expression of TJs. These two features, namely, the vectorial transport and TJ expression, reflect the current physiological understanding of the two-membrane model and define the transporting phenotype characteristic of the BBB endothelium and, in general, all mature epithelia (Fig. 3).

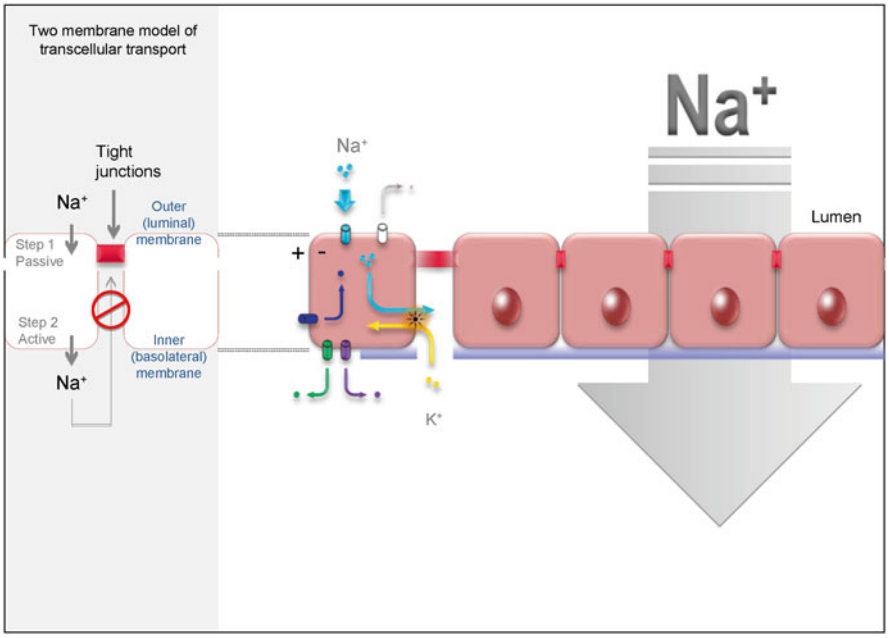

Fig. 3 All endothelia and epithelia share a common transporting phenotype characterized by the expression of tight junctions and vectorial transcellular transport. The classical description of this phenotype established the need of a polarized expression of transporters in the apical (outer/luminal) or basolateral (internal/abluminal) membrane. In order for this model to work as a transporting mechanism, sodium (the most abundant electrolyte in the extracellular fluid) must passively enter the apical membrane, following its concentration gradient, and be actively exchanged with potassium in the basolateral membrane. The sodium pump (Na+/K+-ATPase) is the enzyme that fulfills this role, and its polarized expression is paramount in maintaining the transporting properties of the brain endothelium. In order to prevent sodium backflow, the paracellular space is sealed with tight junctions (*red boxes*). The resulting biochemical effect is the formation of an electrochemical gradient between the intracellular and extracellular sides of the luminal and abluminal membranes. Due to its implications in cell metabolism, this gradient is canonically considered the driving force that allows other membrane transporters to work and ultimately maintain the proper electrochemical environment for cellular metabolism to occur

2.2 Electrical Representation

The introduction of the two-membrane model represented a milestone in understanding the transport characteristics of epithelia and endothelia. However, the observed change in ionic concentration between the apical and basal compartments was not consistent with the total change in voltage across the epithelium/endothelium. This condition was inferred to be caused by transepithelial leakage of ions that could not be explained by the two-membrane model alone, suggesting the existence of an "electrical shunt" pathway. Further studies demonstrated that ionic diffusion through the paracellular space was an important contributor to the electrical shunt [12], and different paracellular conductivities were identified for a number of tissues, leading

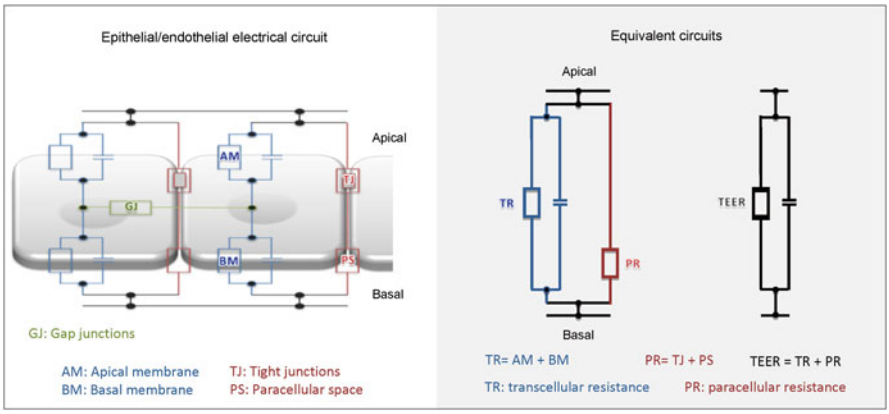

Fig. 4 Electrical gradients arising from the transendothelial ionic transport generate voltage across the apical and basal membranes, tight junctions, and the paracellular space. The particular localization and conductivity of these elements to transport specific ions results in the generation of electrical resistance, creating an electrical circuit with two sets of resistors in parallel: apical and basal membrane (representing transcellular transport), and the tight junctions and paracellular space (representing paracellular diffusion). In addition, direct cell-cell communication via gap junctions allows ions to move between cells, interconnecting adjacent circuits and creating an additional intercellular resistance that has a practical role when the ionic equilibration rate between adjacent cells needs to be accounted for. The transepithelial/transendothelial electrical resistance (TEER) is obtained by obtaining the summatory of the transcellular and paracellular resistances

to the distinction between "tight" (low-conductivity) and "leaky" (high-conductivity) epithelia [13]. It was later proposed that the molecular composition of the TJs was responsible for determining the low or high conductivity of epithelia. These concepts were integrated into the two-membrane model, ultimately representing epithelia/endothelia as an electrical circuit with four resistors: the apical and basal cell membranes form two serial resistors representing the transcellular route, while parallel to them, TJs form a resistor serially coupled to the electrical resistance of the paracellular space, representing the paracellular route (i.e. the shunt pathway) [14] (Fig. 4).

The most sensitive approach to determine the transport function of the BBB endothelium is thus to measure the transendothelial electrical resistance (TEER), which results from the mathematical integration of the transcellular and paracellular resistances. Since TEER is directly proportional to the paracellular resistance, it constitutes an accurate determination of the ionic permeability of TJs.

In general, TJs are selectively permeable to cations, depending on the size and charge; however, their selectivity can be altered by changes in pH, osmotic load, by applying an electrical current [15], or if the molecular structure of the TJ changes. Thus, different ionic selectivities found in different types of epithelia and endothelia depend on the particular molecular composition of TJs [16]. Therefore, it is not surprising that alterations in the molecular composition of TJs of the brain endothelium can influence ionic selectivity and tightness of capillaries influencing the overall BBB permeability.

3 Endothelial Cells and Tight Junctions

Mature endothelial cells are characterized by low height, low number of caveolae in their luminal surface, and numerous mitochondria. Brain endothelial cells (Fig. 5) differ from those outside the brain by the absence of fenestrations, low pinocytic activity, and extensive TJs. The morphology of TJs has been intensively studied by freeze-fracture electron microscopy [17] where it appears as a network of particles organized into multibranched and interconnected fibrils, known as strands. These strands associate with other strands from opposing membranes, forming Velcro-like molecular seal that closes the paracellular space. The number of strands and their branching frequency vary notably among different types of epithelia and correlate with TJ ionic permeability [14] (Fig. 6).

The biochemical nature of TJs started to be characterized when a TJ-enriched membrane fraction obtained from mouse liver was used to generate a monoclonal antibody that detected a previously unknown cytosolic protein of ~225 kDa [18], this was the first TJ protein discovered and named "zonula occludens-1" (ZO-1).

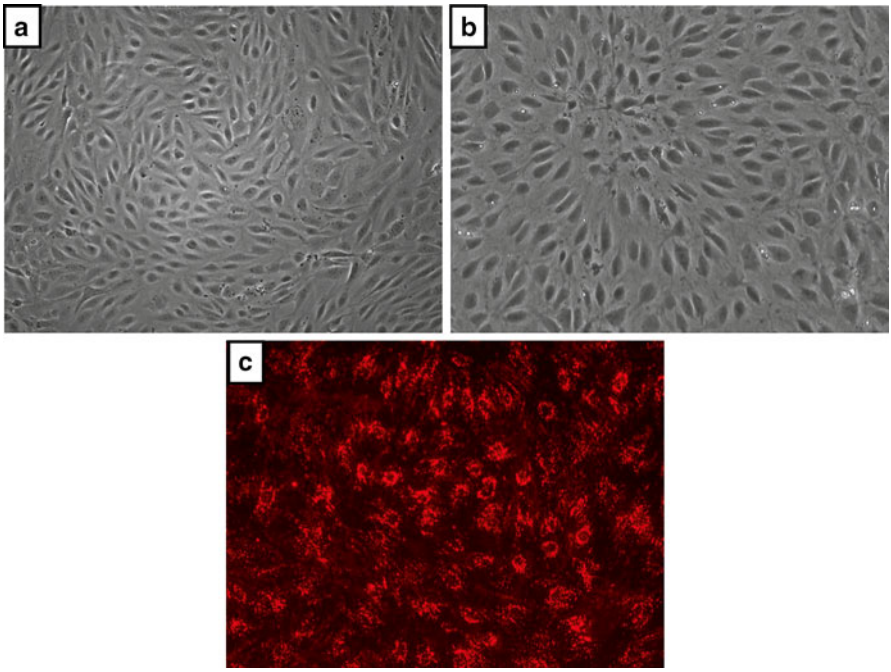

Fig. 5 Brain capillary endothelial cells cultured in vitro. (**a**) Confluent monolayer of frequently used human brain endothelial cells hCMEC/D3. (**b**) Confluent monolayer of primary mouse brain endothelial cells. In (**a**) and (**b**) notice typical morphology of brain endothelial cells, such as a fusiform shape and cobblestone appearance. (**c**) Positive uptake of acetylated low-density lipoprotein are one of the markers of endothelial cells

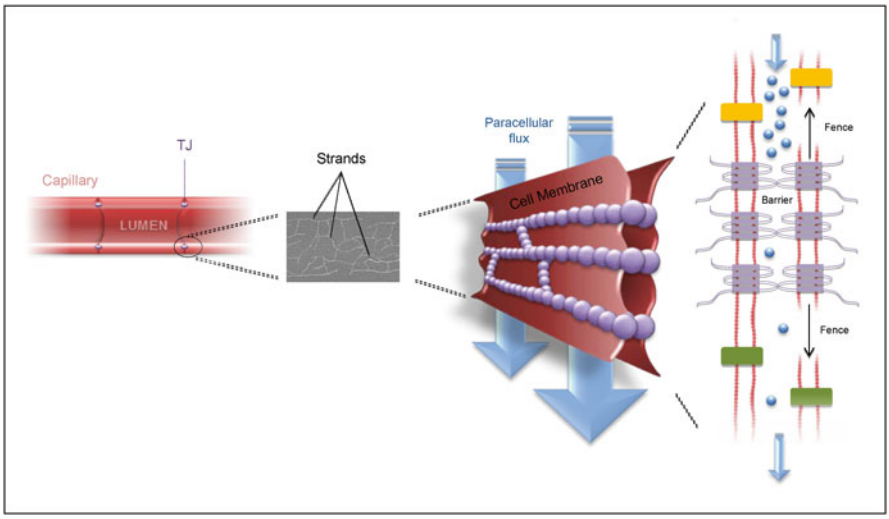

Fig. 6 One of the most prominent features of brain endothelial cells, as well as those from other endothelia or epithelia, is the expression of tight junctions. When studied by freeze-fracture electron microscopy, they appear as a series of filaments, collectively known as strands. Higher number of strands and complex branching patterns have been associated with reduced paracellular ionic permeability (high TEER, i.e. tight paracellular space), whereas fewer strands and simple branching correlate with increased paracellular permeability (low TEER, i.e. leaky paracellular space). These strands are formed by transmembranal proteins that associate laterally in the same membrane (*cis*) to form a fence preventing the free diffusion of proteins and transporters between the apical and basal membrane domains, and frontally with their homologues in a neighboring membrane (*trans*) to form a paracellular barrier that restricts ionic diffusion through the paracellular space

A second molecular component of TJs was later identified as a transmembranal protein of ~65 kDa that received the name "occludin" [19]. Shortly after, two other transmembranal proteins were found claudin-1 and 2, the first of a novel protein family responsible for sealing the intercellular space [20] and forming paracellular ion pores. With the advent of novel protein-protein interaction and molecular screening techniques, the discovery of many additional TJ proteins followed in a short period of time. Currently, more than 40 proteins have been identified as part of TJs [21]. Although the list is large and growing, the key roles in defining the structure and function of TJs reside in a small number of these proteins.

Structurally, TJs consist of a set of transmembranal proteins that interact laterally with each other (*cis*) and across the paracellular space with those expressed in an opposing membrane (*trans*). The *cis*-interaction patterns determine the formation and branching of TJ strands, while the *trans*-interaction arrangements define the paracellular space sealing and formation of ion pores. The transmembranal proteins are scaffolded and attached to the underlying cytoskeleton by a set of submembranal adaptors that, in turn, form a link between transmembranal proteins, regulatory molecules, and transcription factors (Fig. 7).

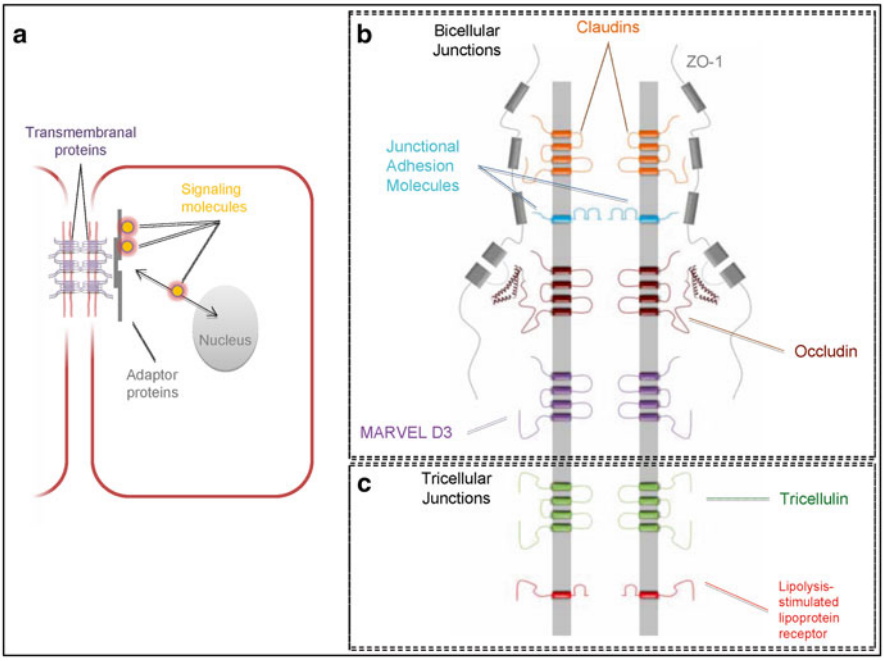

Fig. 7 (**a**) Tight junction complexes are formed by a set of transmembrane proteins anchored and stabilized by a large number of adaptor proteins that, in turn, are associated to the cytoskeleton and numerous signaling molecules that elicit changes in cell metabolism and gene expression. (**b**) The known transmembranal constituents of tight junctions are shown anchored to the submembranal adaptor ZO-1. The figure depicts these proteins in isolation, but they complex to form homo- and hetero-oligomers in living cells. Bicellular contacts are the regions where the membranes of two opposing cells are in contact and the streamlined descriptions of the tight junctions refer to the bicellular junctions. (**c**) A special set of proteins are required in those places where three cells contact each other. Tricellulin and the lipolysis-stimulated lipoprotein receptor organize tight junctions and the tricellular contacts

Epithelia/endothelia are tridimensional structures; therefore, the paracellular space formed where three cells meet together (tricellular contacts) must be subjected to the same sealing and regulatory mechanisms as the bicellular contacts. When the bicellular TJ belt reaches the tricellular contacts, the most apical strands turn down and extend toward the basal membrane, elongating the TJ perpendicularly to the bicellular TJ belt (Fig. 8). Tricellular and bicellular TJs are thus interconnected and form a continuous complex. The vertically oriented strands interact with each other and form an extracellular tubular channel of small diameter (~10 nm) known as the central tube which, similar to the bicellular TJs, regulates paracellular flux (Fig. 8).

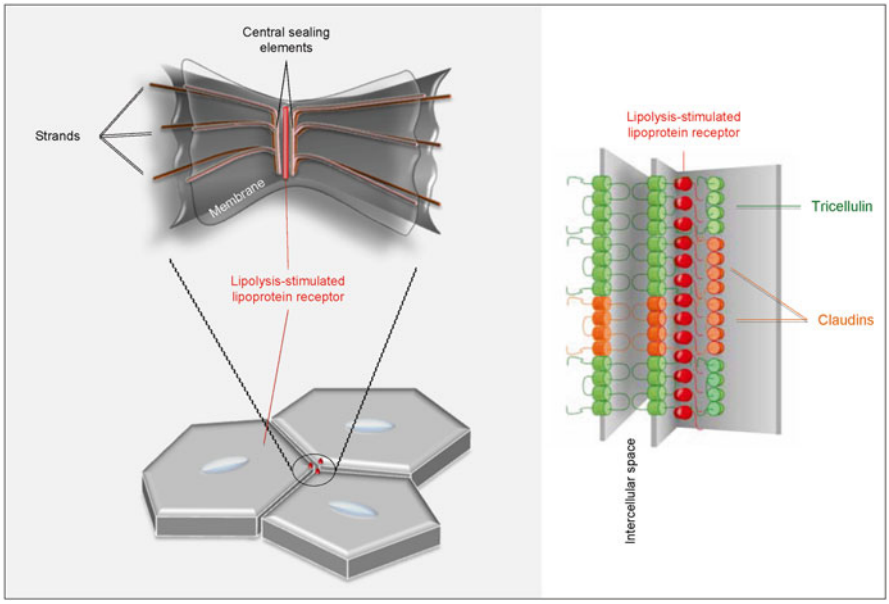

Fig. 8 At the tricellular contacts, the lipolysis-stimulated lipoprotein receptor functions as a beacon for tricellulin to find the cellular corners. Once in place, tricellulin organizes the incorporation of claudin-containing vertical strands to the tricellular borders, forming the central sealing elements. Similar to claudins that form paracellular pores to regulate ionic conductivity, tricellulin controls the ionic environment at the tricellular junctions

3.1 Molecular Structure of the Tight Junctions

3.1.1 Transmembranal Proteins

TJ transmembranal proteins are grouped into three main families: claudins, TJ-associated MARVEL (*m*yelin/lymphocyte *a*nd *r*elated proteins for *ve*sicle trafficking and membrane *l*ink) proteins (TAMPs), and adhesion molecules of the immunoglobulin superfamily. Claudins and TAMPs are tetraspanins, having four transmembranal domains, two extracellular loops, and intracellular N- and C-terminal domains. On the contrary, junctional adhesion molecules (JAMs) are single spanning molecules with a large extracellular domain and a short intracellular tail (Fig. 7).

Claudins

Claudins are the main functional constituents of the TJ strands, seal the paracellular space and form ion pores. Currently, there are 27 known human claudin molecules that, according to their amino acid sequence, can be organized into classic

(claudin-1 to 10, -14, -15, -17, and -19) and nonclassic (claudin-11 to 13, -16, -18, and 20–27) [22]. The precise functional role of many of them is still unknown. Some claudins exhibit a clear paracellular sealing function (claudin-1, -5, -11, and -14), while others are better known for their capacity to form cation pores (claudin-2, -7, -10B, -15, and -16), increasing TJ permeability and decreasing TEER [23]. Claudin-2 is involved in forming pores for monovalent cations (Na^+, K^+, Rb^+, Li^+, Cs^+), claudin-7 and -15 for Na^+, claudin-10B for cations, and claudin-16 for mono- and divalent cations. The cationic permeability is decreased if members of another subset of claudins (claudin-4, -5, -8, -11, -14, and -19) are incorporated into TJs. The formation of anion pores is less understood. Claudin-10A and -7 may be involved in forming Cl^- pores.

Although claudin-1, -3, -5, and -12 are major players in forming TJs between brain microvascular endothelial cells, the involvement (or absence) of other claudin types in the BBB has not been sufficiently clarified. The functions of claudin-6, -9, -12, -13, -17, -18, and 20–27 are still unidentified. Claudin-6, -9, and -13 are thought to be involved in kidney maturation [69]. Claudin-12 and -18 have been found in epithelia and endothelia of the intestine, inner ear, and brain endothelial cells. Claudin-17, -20, -22, and -23 have been found in the kidney, colon, stomach, and placenta, while claudin-21 and 24 have only been identified by genomic analysis.

Different claudins can interact with each other in *cis* and *trans*, homo- and heterotypically. They are anchored in the plasma membrane by adaptor proteins like ZO-1, ZO-2, ZO-3, and the PALS-1-associated tight junction protein PATJ [17, 24]. In most cases, at least two types of claudins are simultaneously expressed; thus, the cell-type-specific variation of claudin isoforms determines the differences in TEER and paracellular permeability [17, 25, 26].

Occludin

Occludin is another constituent of the TJ strands and the first transmembranal protein of TJs to be identified [19]. Although occludin is also a tetraspanin, it does not share similarities with any of the known claudins. It belongs to the TAMP family [27] for which it represents the archetype. It has seven known isoforms generated by alternative splicing. The classical description of occludin refers to the isoform 1, which weighs ~60 kDa, has a very short N-terminal domain, and has a very large C-terminal domain accounting for almost 50 % of its weight. Its MARVEL domain encompasses the four transmembranal domains, the loops in between, and the most N-proximal region of the C-terminal domain. The C-terminal domain has similarities to the RNA polymerase II elongation factor ELL and is thought to mediate protein-protein interactions. Isoform 2 lacks the fourth transmembranal domain, presumably causing the C-terminal domain to become extracellular. Isoform 3 lacks the last 47 amino acids on the C-terminal region, corresponding to half the coiled-coil domain that normally binds to the adaptor protein ZO-1. Isoform 4 is formed by the fourth transmembranal and the full C-terminal domain. Isoform 5 is comprised of the C-terminal domain, excluding a stretch of 57 amino acids on its N-terminal region, and lacks any transmembranal domains, being presumably a cytosolic

protein. Nevertheless, this isoform maintains the full binding site for ZO-1. Isoform 6 is 69 amino acids long, conserves the first 49 amino acids of the N-terminal domain, and presumably is cytosolic. Isoform 7 is 70 amino acids long, conserves the first 50 amino acids of the N-terminal domain, and is also presumed to be cytosolic.

The functions of occludin have proven to be elusive to determine. Most of our current knowledge comes from studying the isoform 1 which has been suggested to be a part of a system that regulates the expression of other TJ molecules and the function of the TJ itself. Occludin may be involved in the activation of the TJ-associated guanine nucleotide exchange factor GEF-H1/Lfc [28] and in targeting the tumoral growth factor-ß (TGF-ß) receptors to the TJs. On the other hand, the interaction of occludin with ZO-1 links it indirectly with other transmembranal TJ molecules (e.g., claudins and JAMs) that also attach to ZO-1. The C-terminal domain is also rich in serines, threonines, and tyrosines that can be differentially phosphorylated [29–31]. In intact cells, occludin is highly phosphorylated on threonine and serine residues, while tyrosines are dephosphorylated. When threonines and serines are dephosphorylated, and tyrosines phosphorylated, occludin delocalizes from TJs. The differential phosphorylation of occludin has been linked to changes in its ability to interact with itself and with ZO-1, as well as modulation of TJ permeability.

Tricellulin

Tricellulin (MARVEL D2) is also a TAMP family member. It is found almost exclusively at the tricellular contacts, where it functions as a master molecule directing organization of the tricellular TJs (Fig. 8). It has an ELL domain and also binds ZO-1 [32, 33]. There are four known isoforms of tricellulin which, similarly to occludin, are formed by alternative splicing. Tricellulin-a is the classical isoform, weighing 64 kDa. Tricellulin-a1 lacks a small number of amino acids in the C-terminal domain, but maintains the ELL domain. Tricellulin-b lacks the ELL domain, and tricellulin-c is predicted to have only two transmembrane domains.

Tricellulin is regulated by phosphorylation, particularly by a PKC-signaling pathway that has been linked to the activity of peroxisome proliferator-activated receptor gamma (PPARγ). The presence of tricellulin is required for the adequate development of TEER and molecular organization of TJ molecules in both tricellular and bicellular contacts. At tricellular contacts, tricellulin selectively seals the paracellular space against macromolecules without altering the ionic permeability of the tricellular TJs [34]. Furthermore, expression of tricellulin has been suggested to protect against viral and transepithelial penetration of foreign antigens. Interestingly, in the absence of occludin in epithelial kidney cells, tricellulin migrates from the tricellular to the bicellular contacts [70], suggesting that tricellulin may partially compensate for some occludin functions. On the other hand, the presence of tricellulin in bicellular TJs reduces strand discontinuities and improves their paracellular barrier function.

MARVEL-D3

MARVEL-D3 is a TAMP member whose association with TJs was recently discovered [35]. There are two known isoforms (~45 and ~46 kDa) widely expressed in epithelia and endothelia. Currently, little is known about its function. MARVEL-D3 expression is not required for functional TJs; interestingly, its depletion results in enhanced TEER. Thus, it has been suggested that MARVEL-D3 modulates the paracellular barrier properties of TJs. MARVEL-D3 was also suggested to have an overlapping function with tricellulin and occludin, although the precise nature of this function needs to be clarified.

Lipolysis-Stimulated Lipoprotein Receptor (LSR)

LSR is a receptor for triacylglyceride-rich lipoproteins that binds chylomicrons and low- and very-low-density lipoproteins in the presence of free fatty acids, allowing their subsequent cellular uptake. It is a single spanning molecule of ~71 kDa with an extracellular N-terminal Ig-like domain, a single transmembranal domain, and a large cytosolic C-terminal domain. There are four known isoforms with slightly different molecular weights. Its recent identification as a TJ molecule led to the proposal of a model that explains the formation of tricellular TJs. LSR is suggested to define a topographic landmark for cellular corners at tricellular contacts [36]. The cytosolic C-terminal domain of LSR binds to tricellulin, suggesting that LSR can be directly involved in recruiting it to the tricellular borders, where in turn, tricellulin directs the organization of the tricellular TJs (Fig. 8).

Junctional Adhesion Molecules (JAMs)

JAMs are members of the immunoglobulin superfamily and, similarly to occludin, are not exclusive to epithelial/endothelial cells, as they are also found in leukocytes and platelets. They are formed by a single transmembranal domain, and their large extracellular domain has two Ig-like motifs. The family is comprised of four members: A, B, C, and 4/L (4 in mouse, L in human). While JAM-A and -C are localized at the TJs, JAM-B is expressed along the whole lateral membrane of endothelial cells.

JAMs are involved in cell adhesion by *trans*-interacting with themselves and integrins; thus, they have been proposed to play a role in adhesion of leukocytes to endothelial cells. Their involvement in TJs also contributes to endothelial barrier function [37], but the mechanisms of such regulation are still not defined [38]. JAM-A, -B, and -C have PDZ-binding motifs that allow them to bind a number of TJ-associated adaptor proteins, such as ZO-1, MAGI-1, or MUPP-1. The serine protein kinase CASK/Lin2 and the cell polarity-related/G protein-coupled receptor Par3 are also known binding partners of JAMs. Therefore, it has been suggested that JAMs have a double role in endothelial cells, regulating leukocyte/platelet/endothelial cell interactions and TJ formation during the acquisition of cell polarity.

Coxsackievirus and Adenovirus Receptor (CAR)

CAR was originally identified as a protein that enables group B coxsackievirus and different types of adenoviruses to attach to the cell surface. It is a single spanning molecule formed by an extracellular N-terminal domain that contains two Ig-like domains, a single transmembrane domain, and a smaller cytosolic C-terminal domain. Five isoforms have been identified, but their functions in TJs are yet to be clarified [39]. CAR-1 has a binding region for ZO-1 and its overexpression leads to an increase in TEER. Since it can bind to IgG and IgM in serum and is overexpressed at sites of inflammation, it has been speculated that, similarly to JAMs, CAR-1 may be involved in the transepithelial transmigration of immune cells.

3.1.2 Submembranal Proteins

These adaptors bind to the transmembrane proteins and allow them to acquire a proper organization in cell membranes, linking them to the cytoskeleton and signaling molecules. Some of these adaptors are big scaffolds (i.e. ZO-1) that allow multiple proteins to be bound simultaneously. The most studied proteins in this group are members of the membrane-associated guanylate kinase (MAGUK) family, MAGUK-inverted proteins (MAGIs), and cingulin.

Membrane-associated Guanylate Kinases

MAGUK proteins are characterized by having one or more PSD95/Disk-large/ZO-1 (PDZ) domains, a Src homology-3 (SH3), and a non-catalytic guanylate kinase homology (GuK) domain. Most of them are scaffolding proteins and localize to cell-cell contacts, where they interact with numerous structural and signaling proteins via their PDZ, SH3, and GuK domains. Based on their size and domain distribution, four MAGUK subfamilies are known: DLG-like, ZO-1-like, p55-like, and LIN2-like. ZO-1-like proteins have the most relevance for TJs. ZO-1, -2, and -3 play a key role in regulating membrane protein assembly, clustering of receptors and ion channels, and regulation of cell differentiation [40].

ZO-1 is a ~220 kDa protein that anchors claudins, occludin, JAMs, and tricellulin in TJs. ZO-1 is thus considered the main TJ scaffolding protein. It also binds the Y-box transcription factor ZONAB, the adherens junction protein β-catenin, the signaling proteins ($G\alpha_{12}$ and $G\alpha_{i2}$), the β-subunit of the L-type Ca^{2+} channel, actin, CAR, afadin, and the desmosomal protein AHNAK [41, 71]. Similar to occludin, expression of ZO-1 is not restricted to TJs and epithelial/endothelial cells. The amino acid sequence of ZO-1 contains nuclear localization signals. In fact, ZO-1 localizes to the cell nucleus in sparse cultures of epithelial or endothelial cells. In brain capillary endothelial cells, the small GTPase Rho is involved in the nuclear localization of ZO-1 by inducing phosphorylation of the transcription factor cAMP response element-binding protein (CREB) [42]. The nuclear localization of ZO-1 has also been observed in proliferating corneal fibroblasts and HEK293T cells.

There are five known isoforms of ZO-1 formed by alternative splicing. The most studied are a+, which corresponds to the classical description, and a−, which lacks 80 amino acids in its C-terminal domain. They appear to have different roles; a+ is predominant in epithelia, while a− is in endothelia. In addition, a+ seems to correlate with the establishment of functional TJs and a− is associated with structurally dynamic TJs that undergo active remodeling, e.g., in Sertoli cells or podocytes [43]. The other isoforms are b1 and b2, about which little is known.

ZO-2 is a 160 kDa molecule that binds to ZO-1, claudins, occludin, cingulin, α-catenin, and actin. In sparse epithelial cultures, it is conspicuously located in nuclear speckles, where it co-localizes with the splicing factor SC35. It also associates with the transcription factors Fos, Jun, and C/EBP. ZO-3 is a 130 kDa protein that binds to ZO-1/ZO-2 complexes and seems to mediate the assembly of TJs by associating with PATJ, cingulin, and occludin [44].

The MAGUK-inverted (MAGI) group of proteins is a subset of MAGUK molecules that, inversely to the archetypical MAGUKs, have most of their PDZ domains located N-terminally to the SH3-GuK domains [40]. MAGI-1 co-localizes with ZO-1 and the GTP exchange protein GEP at TJs. MAGI-2 and -3 form a complex with the phosphatase PTEN that catalyzes the dephosphorylation of phosphatidylinositol 3,4,5-triphosphate, which is involved in apoptosis suppression by activating AKT/PKB.

The protein associated with Lin-7 (PALS-1) is also a MAGUK protein. It is recruited to TJs by the PALS-1-associated tight junction protein PATJ and functions as an adaptor that links PATJ to CRB-1, a molecular scaffold that participates in the development of cell polarity. The resulting PALS1/PATJ/CRB-1 complex plays a paramount role in establishing apico-basal polarity and TJ biogenesis. PALS-1 is also involved in adherens junction formation and the trafficking of E-cadherin.

Other Relevant Proteins

The partitioning-defective protein PAR-3 is associated at the TJ level with JAMs and forms a complex with PAR-6 and the atypicial (a) kinases PKC-λ and PKC-ξ. PAR-6, a binding partner of the Rho GTPases Cdc42-GTP and Rac1, is a key molecule that mediates the association of Rac1, Cdc42, and the atypical PKCs to PAR-3. The PAR-3/PAR-6/Cdc42/Rac1 complex is directly involved in cell polarity during the acquisition of the epithelial phenotype and is thought to be recruited to TJs by the association of PAR-3 with JAMs. PAR-6 also interacts with PALS1, linking PALS1/PATJ/CRB with the PAR3/PAR6/aPKC complex [45].

Afadin, also known as AF-6, is a 205 kDa protein that interacts with ZO-1, cingulin, JAMs, profilin, F-actin, and Fam. The binding between ZO-1 and afadin is mediated by the Ras-binding domains of afadin, and activation of members of the small GTPase family Ras (e.g., Ras, Rap1A, Rit, Rin, and M-Ras) inhibits this binding and disrupts cell-cell contacts [46]. JAMs and ZO-1 are mutually exclusive in their association with afadin, which is a critical regulator of cell-cell junctions during development [47]. Afadin is also located at the adherens junctions, where it binds nectin, a Ca^{2+} independent Ig-like molecule, and ponsin, a molecule that links afadin and

vinculin to adherens junctions. Through its association with profilin, afadin is thought to participate in the cortical actin assembly and cytoskeletal remodeling [48].

The multi-PDZ domain protein 1 (MUPP-1) is a large molecule that is associated, at the TJ level, with claudins, JAMs, and PALS-1 [49]. MUPP-1 is not needed for TJ establishment or polarization, but has instead been proposed to modulate cell proliferation [50].

Cingulin is a ~150 kDa protein that localizes to TJs and interacts with ZO-3, afadin, JAM-A, F-actin and myosin, and forms complexes with ZO-1. Cingulin is known to modulate activity of RhoA by interacting and inactivating its exchange factor GEF-H1, particularly in mature epithelia where it regulates gene expression and cell proliferation [51].

3.2 Transcellular Transport

While endothelial cell TJs limit the paracellular flux of hydrophilic molecules, small lipophilic molecules (e.g., anesthetics, O_2, or CO_2) can diffuse freely across plasma membranes following their concentration gradients. The polarized expression of receptors and transporters embedded in the membranes of endothelial cells, ensures the vectorial transport of nutrients such as glucose and amino acids, and the uptake of larger molecules like insulin or leptin. In endothelial cells, transcellular permeability is largely mediated by endocytic and transcytotic processes [52]. Clathrin plays a major role in the formation of polyhedral lattices that surround and coat endocytotic and transcytotic vesicles. These coated vesicles allow endothelial cells to acquire and transfer nutrients, import signal receptors and growth factors, mediate immune responses, and provide an alternate mechanism to remove pathogens and toxins. However, clathrin-mediated endocytosis can also provide an entry pathway for pathogenic agents.

Whereas conserving a proper barrier function is essential to maintain brain homeostasis, delivery of vital molecules from the blood into the brain is essential to preserve brain metabolism. To achieve this, brain endothelial cells express a large number of transporters in their membranes; however, the expression and activity levels of many of them are controlled by astrocytes [53]. In specific cases a transporter may not be expressed in endothelial cells, as is the case of dopamine, nevertheless, L-DOPA can be transported into the cells, where it is enzymatically transformed to dopamine. Transporters for glucose, galactose, amino acids, monocarboxylic acids, purines, nucleosides, amines, and ions have been identified in the membranes of endothelial cells.

Molecular transport, however, does not occur only from the blood into the brain parenchyma. Non-required metabolites, toxic substances, and drugs must be removed from the brain, and endothelial cells express a large variety of carriers to actively transport these molecules into the blood. P-glycoprotein (Pgp), an ATP-binding cassette (ABC) efflux transporter, is one such major carrier. Along with multidrug resistance-associated proteins (MRPs), Pgp is responsible for the active efflux of a wide range of nonpolar molecules out of endothelial cells.

The plasma membranes of a large variety of cells incorporate microdomains composed of glycosphingolipids and glycolipid-associated proteins. These glycolipoprotein domains, known as lipid rafts, influence membrane fluidity, membrane protein and receptor trafficking, and constitute organizing centers for the assembly of signaling molecules. They have three to five times more cholesterol than other parts of the plasma membranes and are rich in sphingolipids and low in phosphatidylcholine. Because their lipid content is more organized and tightly packed than the surrounding cell membrane, these lipid microdomains float in plasma membranes similarly to a raft in water [54]. Their molecular composition makes them relatively insoluble in nonionic detergents (e.g., Triton X-100) at low temperatures.

Caveolae are small (50–100 nm) lipid rafts, forming invaginations of the plasma membrane induced by the oligomerization of caveolins, of which caveolin-1 forms very high molecular weight oligomers while binding to cholesterol and fatty acids. Caveolae have functional effects on signal transduction and play a role in endocytosis, oncogenesis, and the uptake of pathogenic bacteria and viruses [55]. They have been suggested to be a docking site for glycolipids and glycosylphosphatidylinositol-linked proteins. Caveolae contain numerous receptors and transporter systems, including receptors for low- and high-density lipoproteins, insulin, albumin, transferrin, advanced glycation end products, ceruloplasmin, interleukin-1, and vesicle-associated membrane protein-2 (VAMP-2). Caveolin-1, besides its role in the structural arrangement of caveolae, forms signaling complexes with endothelial nitric oxide synthase (eNOS), heterotrimeric G proteins, members of the membrane-associated protein kinase (MAPK) pathway, src tyrosine kinase, and protein kinase C. Caveolin-1 also regulates TJ protein expression. For example, cytoskeletal rearrangements due to actin depolymerization can cause TJ proteins to be internalized by caveolae-mediated endocytosis [56].

4 Astrocytes

Astrocytes are the most abundant cell type in the human brain and one of the major types of glial cells. They are morphologically characterized by star-shaped bodies and histologically identified by their content of intermediate filaments constituted by glial fibrillary acidic protein (GFAP) (Fig. 9). They express a large number of G-coupled receptors, which transduce neurotransmitter, neuromodulator, and hormonal signals into Ca^{2+} and cAMP signaling cascades. According to their morphology, astrocytes are classified as fibrous, protoplasmic, or radial. Fibrous astrocytes are found mainly in the white matter, contain a small number of organelles, and have many long and unbranched fiber-like processes. Protoplasmic astrocytes are the most abundant and localize in the gray matter, they contain a larger quantity of organelles and exhibit fewer and relatively shorter multibranched processes. Radial astrocytes are located perpendicular to the axis of the ventricular system, having one of their processes immersed into the pia mater and the other buried in the gray matter.

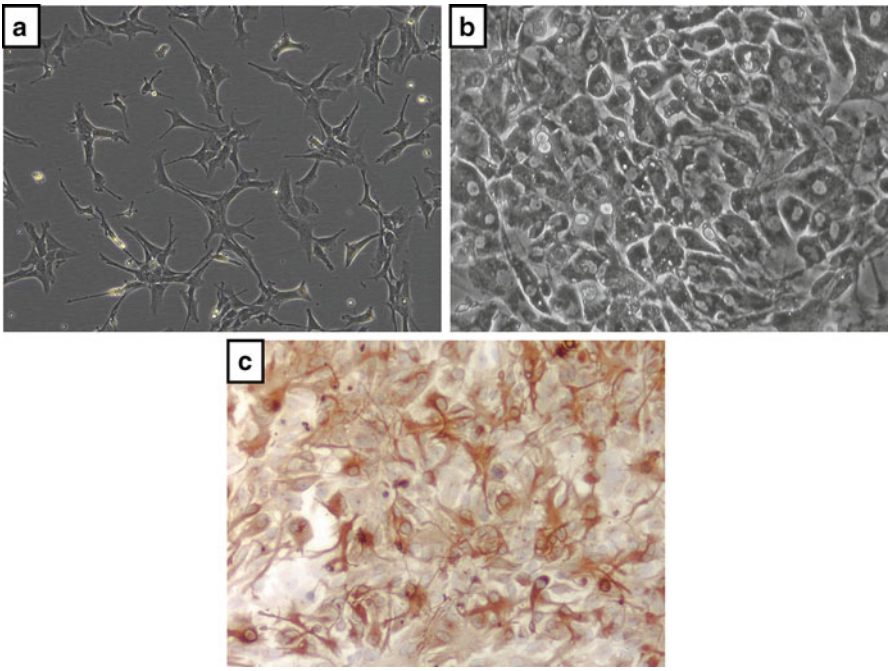

Fig. 9 Cortical astrocytes cultured in vitro and visualized by light microscopy. (**a**) Spare culture of human astrocytes; notice the characteristic star-shaped morphology. (**b**) Confluent culture of mouse astrocytes isolated from neonatal mice revealing typical features of confluent cultures, such as multipolar shape and cell overlapping. (**c**) GFAP-positive immunoreactivity of a confluent culture of mouse astrocytes

They are involved in neuronal migration during CNS development but decrease in number in the adult brain [57]. Regardless of their type, all astrocytes emit processes (vascular feet) that make extensive contact and surround the neighboring capillaries; however, fibrous astrocytes contact the nodes of Ranvier, while their protoplasmic counterparts envelop the synapses. Gap junctions are found in the distal regions of their processes, enhancing astrocyte-astrocyte communication directly in these regions. When in close proximity to the pia mater, astrocytes also emit processes towards it, forming the pia-glial membrane.

Astrocytes populate the CNS in a well-organized and nonoverlapping manner. Under normal conditions, protoplasmic astrocytes maintain well-limited and nonoverlapping domains in the gray matter in such way that only the most distal region of their end-feet interdigitates with one another. Similar domain organization seems to exist in the white matter. There is a considerable structural and molecular diversity among astrocytes at local and regional levels [58], and the complexity and diversity of astrocytes associated with neurons seem to have increased in different

species at different evolutionary stages. In the human brain, an average of 1.4 astrocytes per neuron in the cortex has been proposed [59].

Functionally, astrocytes express potassium and sodium channels, can exhibit evoked inward currents (but cannot trigger or propagate action potentials along their bodies), and show controlled fluctuations in their intracellular calcium concentration that are related to astrocyte-astrocyte and astrocyte-neuron communication. These fluctuations result from calcium release from intracellular stores triggered by neurotransmitters and can, in turn, induce the release of neurotransmitters (e.g., glutamate) from astrocytes into the extracellular space and trigger neuronal activity. Calcium signaling can also propagate to neighboring astrocytes via gap junctions, eliciting responses across their large intercommunicated cellular network. As gap junctions exist between astrocytes, pericytes, and endothelial cells, astrocyte signaling can elicit effects at the capillary level in their respective topographical domains. In fact, astrocytes produce and release various molecular mediators like prostaglandins, nitric oxide, and arachidonic acid that can increase or decrease vessel diameter and thereby control blood flow and intravascular hydrostatic pressure.

The astrocytic end-feet that surround all synapses maintain the homeostasis of the synaptic interstitial fluid. This is important as proper ionic concentration and pH are paramount for synaptic transmission. End-feet have a high content of potassium influx transporters and a number of proton-transporting mechanisms such as Na/H exchanger, HCO_3 and monocarboxylic acid transporters, and the vacuolar-type H-ATPase. GABA, glycine, and glutamate transporters are also enriched in these areas and contribute to clear the synaptic space of these neurotransmitters. Once retrieved, neurotransmitters are interconverted into other metabolites and send back to the synaptic space for their recycling into active transmitters. The large astrocytic networks are responsible for the uptake of potassium and glutamate from the synaptic spaces, preventing the deleterious effect of their synaptic accumulation. Astrocytes can also modulate synaptic transmission by releasing synaptic active molecules such as glutamate, purines (i.e., ATP and adenosine), GABA, and D-serine, in response to changes in synaptic activity. They also produce neurosteroids like estradiol and progesterone and release growth factors that can have long-term effects by influencing synaptic remodeling [60].

On the capillary side, end-feet-contacting endothelial cells are enriched in aquaporins, in particular aquaporin-4. This water transporter regulates water content in the paracellular space and has special clinical relevance since its altered function plays a role in the formation of brain edema [62]. Glucose transporters are also expressed in this location, and glucose transport is elicited upon glutamate stimulation [63]. Glucose can be taken from endothelial cells and extracellular space and is stored as glycogen. In fact, astrocytes constitute the main glycogen storage in the CNS, and the density of astrocytic glycogen granules correlates with synaptic density. Astrocyte-derived glycogen can sustain neuronal activity during hypoglycemia and is used when high neuronal activity occurs. Both the content of glycogen in astrocytes and the gap junction-mediated exchange of glucose metabolites are regulated by neuronal activity (Fig. 10).

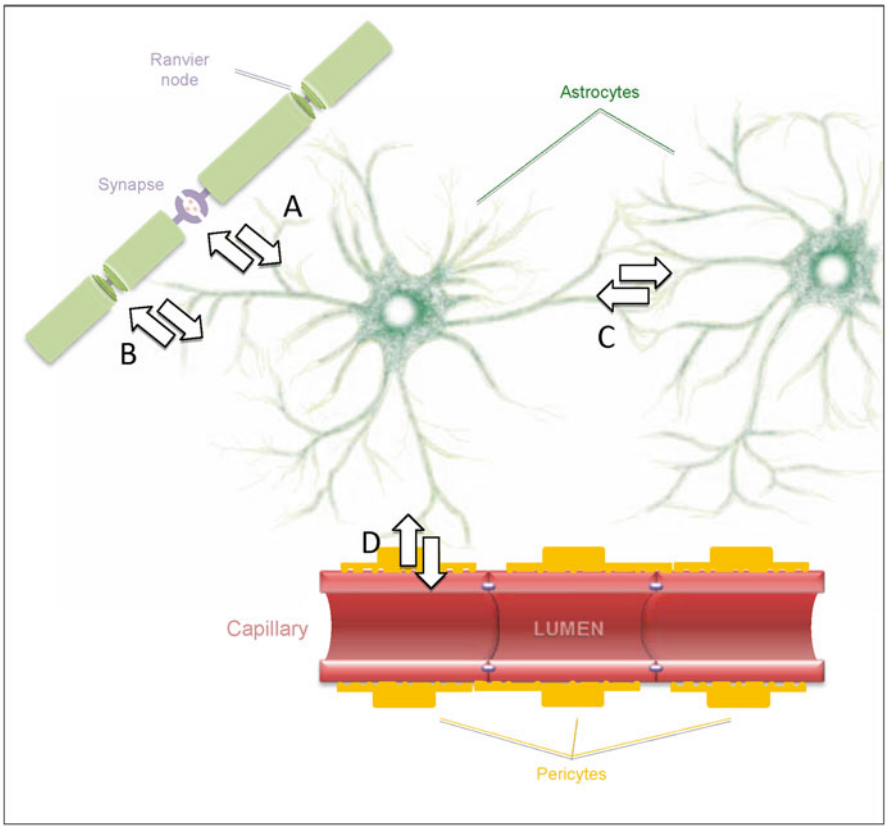

Fig. 10 Astrocytes play paramount roles in maintaining the homeostasis of the brain parenchyma, synaptic function, and transcapillary transport. (*A*) At synapses, their end-feet release energy substrates such as lactate, precursors for neurotransmitters, and neurotransmitters such as glutamate, purines (e.g., ATP or adenosine), growth factors, and neurosteroids. They also remove potassium, water, and neurotransmitters like GABA, glutamate, and glycine. (*B*) At the nodes of Ranvier, astrocytes also exchange energy substrates and electrolytes. (*C*) The astrocyte end-feet are rich in gap junctions that allow the exchange of neurotransmitters, glucose, and ions from one cell to the other and ultimately transport them between the brain capillaries and neurons, or across different astrocyte territories. (*D*) At the capillary level, astrocytes take up glucose and water and release ions, nitric oxide, and amino acids influencing blood flow

5 Pericytes

Pericytes are cells located in the perivascular space and wrapped around endothelial cells to which they provide structural support while conferring vasodynamic capacity to the capillaries. They are also necessary for BBB maturation and maintenance of its properties as they support angiogenesis and prevent endothelial cell apoptosis [64]. Capillary vasoconstriction was the first function identified for pericytes [65]

and can occur in response to vasoactive substances and neurotransmitters. This function has implications for brain activity, as increased local neuronal depolarization is accompanied by incremented regional blood flow. Biochemically, pericytes express a number of receptors for chemical mediators like catecholamines, angiotensin II, vasoactive intestinal peptides, endothelin-1, and vasopressin.

The precise structural and molecular identity of pericytes is still controversial. There is also controversy considering their cell lineage, as pericytes are often classified together with periendothelial smooth muscle cells, fibroblasts, macrophages, sometimes even confused with even endothelial cells. There are no known molecular markers that can be used to specifically identify pericytes and distinguish them from other mesenchymal cells. Furthermore, markers that are commonly found in pericytes are not stably expressed. Consequently, pericytes are often defined by considering their perivascular location, morphology, and gene expression patterns (Fig. 11).

In the BBB, pericytes are in direct contact with the abluminal side of endothelial cells. They extend cytoplasmic processes that often span several endothelial cells and occasionally bridge between neighboring capillary branches where the main body of pericyte resides directly at the branching point and its cytosolic processes extend along each branch. Their density in the human CNS is approximately one pericyte per 1–3 endothelial cells. Pericytes cover approximately 30 % of the abluminal surface of endothelial cells [66, 67]. At the molecular level, platelet-derived growth factor receptor-beta, chondroitin sulfate proteoglycan-4 (NG2), CD13, and alpha-smooth muscle actin are consistently expressed in pericytes [64], although these markers are also found in other mesenchymal cells.

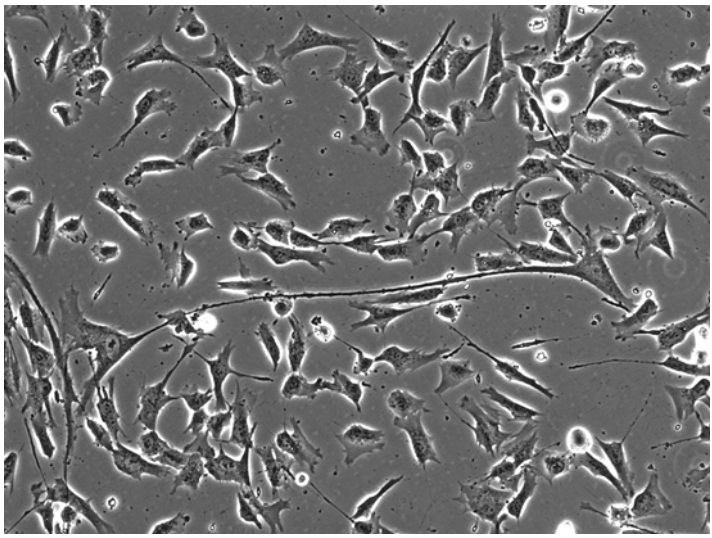

Fig. 11 Spare culture of human brain microvascular pericytes visualized by light microscopy

5.1 Extracellular Matrix

The extracellular matrix (ECM) in the CNS has a distinct composition and organization than that found in other tissues. Since the brain is mechanically protected and enjoys a physicochemically privileged location, there is no need to maintain the high levels of tensile or elastic strength usually present in other tissues. Therefore, the brain ECM, which is predominantly represented by the vascular basal membranes and meninges, has very little amounts of collagen types I and III or fibronectin and has a reduced content of glycosaminoglycan-proteoglycans and hyaluronan. Instead, the thin (20–200 nm thick) and tightly interwoven layers of ECM are predominantly composed of laminins, collagen type IV, heparan sulfate proteoglycans, and nidogens, all of them occurring in different isoforms that generate an ECM with distinct biochemical and functional properties [68]. Laminins in the endothelial cell basal membrane contain chains a4 and a5 combined with b1 and g1 to respectively form the isoforms 411 and 511, while the outer parenchymal basal membranes found at the postcapillary vessels and venules have chains a1 and a2 combined with b1 and g1 to form laminins 111 and 211. Laminins a4 and a5 are produced by endothelial cells, laminin a1 is produced by the leptomeningeal cells and laminin a2 is produced by the astrocyte end-feet. The predominant heparan sulfate proteoglycan in endothelial cell basal membranes is perlecan, while agrin is the predominant counterpart in the parenchymal ECM [61]. Endothelial cell membranes also have additional components such as osteonectin; fibulin-1 and -2; collagen VIII, XV, and XVIII, and thrombospondin-1 and -2. At the capillary level, the endothelial and parenchymal basal membranes are combined and contain laminins a2, a4, and a5, perlecan, and agrin.

6 Conclusions

The BBB constitutes one of the most impregnable mammalian interfaces and is responsible for maintaining the brain homeostasis. It is composed by closely interacting functional multicellular units called the neurovascular units. The properties of the BBB are defined by the unique characteristics of brain capillary endothelial cells that are sealed together by TJ proteins. TJ complexes are formed by transmembranal proteins closely interacting with specific anchor and adaptor proteins. The permeability of the BBB can be modulated by various pharmacological interventions. Importantly, disruption of the BBB integrity is associated with numerous chronic and acute CNS disorders, in which it contributes to the development of neuroinflammatory changes.

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