Neural Membranes and Barriers
Abstract: The blood–brain barrier (BBB) protects the neural microenvironment from changes of the blood composition. It is located in the endothelium, which is both seamless and interconnected by tight junctions. The restrictive paracellular diffusion barrier goes along with an extremely low rate of transcytosis and the expression of a high number of channels and transporters for molecules that cannot enter or leave the brain paracellularly.

Many tight junction molecules have been identified and characterized including claudins, occludin, zonula occludens protein-1 (ZO-1), ZO-2, ZO-3, cingulin, 7H6, junctional adhesion molecule (JAM), and endothelial cell-selective adhesion molecule (ESAM). Signaling pathways involved in tight junction regulation include G-proteins; serine, threonine, and tyrosine kinases; extra- and intracellular calcium levels; cAMP levels; proteases; and cytokines. Most of these pathways modulate the connection of the cytoskeletal elements to the tight junction transmembrane molecules. Additionally, cross talk between components of the tight junctions and the adherens junctions suggests a close functional interdependence of the two cell–cell contact systems.

The BBB endothelial cells are situated on top of a basal lamina, which contains various molecules of the extracellular matrix. Pericytes and astrocytes directly contact this basal lamina; however, little is known about the signaling pathways between these cell types and the endothelium, which possibly are mediated by components of the basal lamina. To analyze the interplay between astrocytes, pericytes, the extracellular matrix, and the endothelial cells is a big challenge for understanding the BBB in health and disease.

1 Introduction

The original finding of Ehrlich (1885) that an infused dye did not stain the brain tissue, together with the complementary observation of his pupil Ernst Goldmann that the very same dye if applied into the cerebrospinal fluid did stain the brain tissue, has lead to the concept of a biological barrier between blood and brain. Due to the free access of the dye from brain ventricle to brain tissue, it was concluded that there is no cerebrospinal fluid–brain barrier. However, the staining of circumventricular organs (CVO) and the choroid plexus, when a dye was applied into the general circulation (Goldmann-I-experiment), and the avoidance of staining of these organs, when a dye was applied into the cerebrospinal fluid (Goldmann-II-experiment), suggested the existence of a barrier between the cerebrospinal fluid and the blood. The cellular basis of these barriers was unclear for decades. Today, we know that in most vertebrates the barrier is located within the endothelium (endothelial blood–brain barrier (BBB); only in elasmobranchs, the BBB is located in astrocytes) and in the epithelial choroid plexus cells and the tanyocytes of the CVO (glial blood–cerebrospinal fluid barrier (BCSFB) Figure 1-1).

The structure restricting the paracellular permeability is the tight junction, and this structure implies the necessity of transporters for substances that must overcome the barrier for providing the brain with energy-rich substrates. The BBB is only one part of a huge regulatory neurogliovascular machinery, which controls the blood flow and the delivery of oxygen and substrates to the brain according to continuously changing local requirements. The endothelial BBB is regulated by many more factors than all other endothelial cells outside the central nervous system (CNS). The reason for that is the enormous complexity of the consciousness producing brain and the complete dependency from blood perfusion. This situation makes it very difficult to analyze the network of causal relationships between the different components of the neurogliovascular complex. This brief overview tries to follow some lines of evidence that astroglial cells, together with the extracellular matrix between glial endfeet and the endothelium, manage the barrier properties of the BBB, which is primarily established by tight junctions under the control of the brain microenvironment.

2 The Astrocytes Inducers of the Blood–Brain Barrier

It is now generally accepted that the astrocytes play a decisive role in the maintenance if not induction of the BBB (Janzer and Raff, 1987; Raub, 1996; Abbott, 2002; Brillault et al., 2002; Engelhardt, 2003; Wolburg and
Lippoldt, 2002; Lee et al., 2003; Begley and Brightman, 2003). This concept came up along with experiments showing that astrocytes placed adjacent to endothelial cells in vitro or in vivo supported the development of barrier properties in the endothelial cells. Among the barrier properties tested in these experiments were transendothelial electrical resistance, paracellular permeability of electron-dense tracers, and the expression of barrier-related molecules. Although in vitro models of the BBB have the principal disadvantage of not being able to simulate the whole of the microenvironmental complexity of the brain, they frequently were successful in investigating regulatory mechanisms concerning glio–vascular interactions (Arthur et al., 1987; Méresse et al., 1989; Rubin et al., 1991; Tontsch and Bauer, 1991; Abbott et al., 1992; Wolburg et al., 1994; Stanness et al., 1999; Franke et al., 2000; Gaillard et al., 2001; Cucullo et al., 2002; Nitz et al., 2003; Parkinson et al., 2003). Hamm et al. (2004) demonstrated an increased transendothelial permeability for horseradish peroxidase (HRP) after discontinued coculture with astrocytes, but this change in permeability was not paralleled by a change in tight junction protein expression. The authors concluded that loss of localization of tight junction associated proteins from the BBB tight junctions might be a relatively late event, which is not yet observed in the comparatively short-term in vitro experiments. In vivo, a reversible disruption of tight junctions has been observed after transitory loss of astrocytes as induced by a single dose of 3-chloropropanediol (Willis et al., 2004). Loss of astroglia-derived glial fibrillary acidic protein (GFAP) went along with the fragmentation of occludin immunoreactivity. This observation was in line with experiments performed in GFAP-deficient mice in which an impaired BBB in vivo (Liedtke et al., 1996) or the failure of astrocytes from GFAP-deficient mice to induce BBB properties in aortic endothelial cells in vitro have been described (Pekny et al., 1998). Unfortunately, although close examination of the spatial relationship of glial cells and the expression pattern of GFAP made it tempting to speculate that cellular interactions between neurons, GFAP-expressing glial cells, and endothelial cells supported the establishment of barrier properties in endothelial cells (Gerhardt et al., 1999a), the mechanism of how GFAP-expressing glial cells perform this task is still completely unknown.

In an approach to identify factors that are involved in the induction of BBB properties, the glial cell line–derived neurotrophic factor (GDNF) was found to be successful in BBB induction (Igarashi et al., 1999; Utsumi et al., 2000; Yagi et al., 2000). The src-suppressed C-kinase substrate (SseCKS) in astrocytes...
has been reported to be responsible for the decreased expression of the angiogenic permeability factor vascular endothelial growth factor (VEGF) and the increased release of the antipermeability factor angiopeitcin-1 (Ang-1). SSeCKS overexpression increased the expression of tight junction molecules and decreased the paracellular permeability in endothelial cells (Lee et al., 2003).

An interesting correlation exists between astroglial differentiation and BBB maturation. The astroglial cells form processes to many compartments of the CNS including synapses, Ranvier nodes, and neural–mesenchymal borders at the surface of the CNS and around vessels. They are extremely rich in gap junctions that not only connect processes of different astrocytes, in particular, in the region of superficial and perivascular endfeet (Figures 1-1 and 1-2), but also different domains of the identical cell (Wolff et al., 1998). The network of neuroglivascular interactions (the “neurovascular unit”) is involved in manyfold metabolic dependencies between neurons and glial cells on the one hand and glial cells and vascular cells on the other hand (for a comprehensive overview on the neurovascular unit, see Iadecola, 2004). The direct interface between the neuroglial compartment and the vascular compartment is established by the perivascular glial endfeet, forming the glial limiting border (Figures 1-1, 1-2, and 1-4a).

The membranes of astroglial endfeet are characterized by a very special molecular architecture. They not only carry plenty of transporters and ion channels but also the dystrophin–dystroglycan complex (Blake and Kröger, 2000), the water channel protein aquaporin-4 (AQP4) (Amiry-Moghaddam et al., 2004), the so-called orthogonal arrays of particles (OAPs) (Figures 1-2, 1-3b, and 1-4) (Wolburg, 1995a), and a recently identified member of the immunoglobulin superfamily, limitrin (Yonezawa et al., 2003).

One of the most important physiological functions of astrocytes is the maintenance of the extracellular K⁺ concentration after neuronal excitation (Orkand et al., 1966). In a series of classical experiments, Newman found in the retinal Müller cell as a model system of astroglial cells that the K⁺ conductivity across the surface of the cell is heavily concentrated where the cell contacts the perivascular or the superficial glial limiting membrane (as reviewed in Newman and Reichenbach, 1996; Kofuji and Connors, 2003). The principle of spatial buffering (in the brain) or siphoning (in the retina) means that K⁺ efflux takes place spatially apart from the site of K⁺ influx effectively stabilizing the extracellular K⁺ concentration. Later on, the inwardly rectifying K⁺ channel, Kir4.1, was identified as one important member of K⁺ channels, which
is highly concentrated at glial endfeet membrane domains and responsible for spatial buffering (Kofuji et al., 2002; Kofuji and Newman, 2004). In rodents, the K\(^+\) channel is expressed in astrocytes surrounding synapses and perivascular endfeet around blood vessels (Higashi et al., 2001; Li et al., 2001a, b) (Figure 1-3).

Interestingly, the distribution of the Kir4.1 channel protein and the K\(^+\) conductivity is similar to that of the dystrophin–dystroglycan complex and the water channel protein AQP4 (Blake and Kroger, 2000; Amiry-Moghaddam and Ottersen, 2003; Connors et al., 2004; Nagelhus et al., 2004; Warth et al., 2005). In contrast, within the neuropil the parenchymal astroglial membranes do express these molecules to an essentially less extent. The polarization of astrocytes, which is related to the distribution of OAPs and the K\(^+\) conductivity, arises concomitantly with the maturation of the BBB (Wolburg, 1995b; Nico et al., 2001; Brillault et al., 2002; Yonezawa et al., 2003; Nicchia et al., 2004). Regarding the OAPs, it is well-known that they contain at least the water channel protein AQP4 (Figure 1-4). Aquaporins mediate water movements between the intracellular, interstitial, vascular, and ventricular compartments, which are under the strict control of osmotic and hydrostatic pressure gradients (Badaut et al., 2002; Papadopoulos et al., 2002). The involvement of AQP4 in the OAP formation was demonstrated by the absence of OAPs in astrocytes of the...
AQP4-deficient mouse (Verbavatz et al., 1997), by formation of OAPs in chinese hamster ovary cells stably transfected with AQP4 cDNA (Yang et al., 1996), and by the immunogold fracture-labeling technique showing that AQP4 is a component of the arrays (Rash et al., 1998, 2004). Moreover, Nielsen et al. (1997) were able to demonstrate by immunogold immunocytochemistry that the distribution of the AQP4-related immunoreactivity was identical to that of the OAPs. It should be stressed that AQP4 is the only member of the aquaporin family, which is associated with a membrane structure demonstrable by electron microscopy (Figure 1-4).

Under brain tumor conditions, the density of OAPs of astroglioma cells has been demonstrated to decrease (Neuhaus, 1990). However, the AQP4 content as detected by immunocytchemistry was increased (Saadoun et al., 2002) and the localization no more restricted at the perivascular endfeet but redistributed across the whole surface of the cell (Warth et al., 2004). These apparently conflicting findings can only be resolved, if one suggests that under glioma conditions AQP4 exists separated from the OAP in the membrane and is no more restricted to the glial membranes contacting the basal membrane. There was a positive correlation between AQP4 restriction at the endfoot membrane (polarization of the astrocyte) and presence of agrin in the vessel basal lamina (Warth et al., 2004). Importantly, the heparan sulfate proteoglycan agrin, an extracellular matrix component, does not bind to AQP4 but to α-dystroglycan.
vasculature (Angelov et al., 1998; Balabanov and Dore 2002) (Figure 1-2 and Figure 1-4a), the small number of caveolae at the luminal surface of the cell (Peters et al., 1991), and the large number of endothelial mitochondria (Coomber and Stewart, 1985). In addition, subendothelial pericytes that are completely surrounded by a basal lamina, phagocytic cells, the interendothelial tight junctions (Brightman and Reese, 1969; for a review, see Wolburg and Lippoldt, 2002) (Figure 1-2 and Figure 1-4a), the small number of caveolae at the luminal surface of the cell (Peters et al., 1991), and the large number of endothelial mitochondria (Coomber and Stewart, 1985). In addition, subendothelial pericytes that are completely surrounded by a basal lamina, phagocytic perivascular cells, and astrocytic processes belong to the set of elements directly adjacent to the cerebral vasculature (Angelov et al., 1998; Balabanov and Dore-Duffy, 1998; Gerhardt and Betsholtz, 2003).

The microvascular endothelial cells are doubtless most important in the restriction of the BBB-related permeability. During brain angiogenesis and differentiation of the BBB, specific molecules have to be expressed in brain endothelial cells. Specific expression of the nonreceptor tyrosine kinase lyn (Achen et al., 1995) and gene products encoding P-glycoprotein have been demonstrated early during brain angiogenesis (Qin and Sato, 1995). Whereas the significance of lyn expression during brain angiogenesis is not clear so far, expression of P-glycoprotein is required for the differentiation of the BBB (Schinkel et al., 1994) and seems to ensure the rapid removal of toxic metabolites from the neuroectoderm before the BBB has fully differentiated (Begley, 2004). In the developing chicken CNS, it has been shown that angiogenic vessels invade the neuroectoderm express N-cadherin between endothelial cells and pericytes. With the onset of barrier differentiation, N-cadherin labeling decreased suggesting that transient N-cadherin expression in endothelial and perivascular cells may represent an initial signal, which may be involved in the commitment of early blood vessels to express BBB properties (Gerhardt et al., 1999b). The early adhesion between endothelial cells and pericytes might be the result of the release of chemotactic factors by endothelial cells to induce the migration of pericytes toward the endothelial cell wall and subsequent maturation of the vessels by an increased production of extracellular matrix components elicited by the action of activated TGF-β and other proteins (Folkman and D’Amore, 1996). Among these, platelet-derived growth factor (PDGF)-B, a high-affinity ligand for the receptor tyrosine kinase PDGF-Rβ present on pericytes is produced by endothelial cells during development. PDGF-B has been shown to be involved in vascularization of the brain as disruption of the PDGF-B gene leads to pericyte loss, endothelial hyperplasia, and lethal microneurysm formation during late embryogenesis (Lindahl et al., 1997). The fine structural investigation of endothelial cells in these PDGF-B-deficient and PDGF-Rβ-deficient mice showed a malformation of brain endothelial cells characterized by the folding of the luminal surface (Hellström et al., 2001). This increase in the luminal surface is also a typical feature of the blood vessels of the pecten oculi that is a convolute of vessels within the vitreous body of the avian eye. In these vessels, the pericytes die by apoptosis during development (Gerhardt et al., 2000). Thus, both the physiological loss of pericytes in the pecten oculi...
and the pathological loss of pericytes in the PDGF-Rβ-deficient mouse lead to a characteristic alteration of the shape of endothelial cells, suggesting a role for pericytes in the morphogenesis of microvessels.

Establishing the barrier is accompanied by further changes in the phenotype of the brain endothelial cells, such as upregulation of the HT/7-antigen/basigin (Seulberger et al., 1992) or downregulation of the MECA-32 antigen (Hallmann et al., 1995), besides the expression of specific transporters, and metabolic pathways can be observed (Pardridge, 1988, 1991; Mann et al., 2003). As far as the the development of the barrier function of brain capillaries is concerned (Wakai and Hirokawa, 1978; Risau, 1991; Stewart, 2000; Engelhardt, 2003), it has become clear that BBB tightness is not just “switched on” at a specific time point during brain angiogenesis but rather the tightening of the barrier occurs as a gradual process, which is independent from vascular proliferation and begins late during embryogenesis when angiogenesis is not complete (Risau et al., 1986). The molecular mechanisms involved in this process are yet poorly understood. From transplantation studies showing that vessels derived from the coelomic cavity gain BBB characteristics when growing into an ectopic brain transplant (Stewart and Wiley, 1981), it is known that the development of BBB characteristics in endothelial cells are not predetermined but rather induced by the neuroectodermal microenvironment.

4 The Structure of Tight Junctions

The notion that tight junctions form an efficient permeability barrier originally came from electron microscopic tracer experiments (Reese and Karnovsky, 1967; Brightman and Reese, 1969). After intravascular perfusion of an electron-dense tracer, such as HRP or lanthanum nitrate, the diffusion of the tracer stopped where tight junctions interconnected endothelial cells or processes. The most important cells responsible for the establishment of the barrier are the capillary endothelial cells in case of the BBB (Figures 1-1, 1-2, and 1-4a) and the epithelial (glial) cells in case of the BCSFB (Figure 1-1).

In endothelial cells, these specialized contact zones were already known from ultrathin sections (Muir and Peters, 1962), and in epithelial cells, their morphology was described in detail by Farquhar and Palade (1963). Around the same time when the endothelial nature of the BBB was detected by Reese and Karnovsky (1967) and classically described in the comprehensive study of Brightman and Reese (1969), the freeze-fracturing technique was developed which allowed the cleavage of cytoplasmic membranes. Soon after its introduction, tight junctions were the issue of manyfold freeze-fracture descriptions (as summarized by Staehelin, 1974), including endothelial cell tight junctions of the BBB (Dermietzel, 1975a, b; Tani et al., 1977; Shivers, 1979; Nagy et al., 1984; Mollgard and Saunders, 1986; Wolburg et al., 1994).

If sectioned transversally in ultrathin sections, the tight junction appears as a system of fusion (“kissing”) points, each of which represents a sectioned strand. If the tight junctional belt running around the apical circumference of the cell is broad in the apico-basal direction, the number of kissing points in ultrathin sections will be larger as if the belt would be narrow. If cleaved by freeze-fracturing, the tight junctions are visualized directly as a network of strands within the fracture plane of the apical membrane (Figure 1-4c, 1-4d). Regarding this network, two parameters can be recognized: the complexity of strands and the association of the particles with the inner (P-face) or outer (E-face) lipidic leaflet of the membrane. The complexity of the tight junction network could be described as a logarithmic relationship between the number of tight junction strands and the transcellular electrical resistance (Claude, 1978). Concerning the P-face/E-face association, epithelial tight junctions are mostly associated with the P-face, forming a network of strands, leaving grooves at the E-face, which are occupied by only few particles (Martinez-Palomo et al., 1980; Madara and Dharmsathaphorn, 1985). After adenosine triphosphate (ATP) depletion, Madin-Darby canine kidney (MDCK) cells suffer from deterioration of the paracellular barrier (“gate”) function, which is accompanied by a reorganization of the actin cytoskeleton (Mandel et al., 1993; Bacallao et al., 1994) and a decreased P-face association of the tight junctions. Taken together, the network complexity as well as the degree of particle association to the P-face seems directly to correlate with the observed transepithelial resistance.

The freeze-fracture structure of the brain capillary endothelial cell tight junctions was investigated by Nagy et al. (1984). The authors found the brain endothelial cell tight junctions most complex in the
whole vasculature of the body. In addition, the BBB tight junctions were described to be unique among all endothelial tight junctions in that their P-face association is as high as or even slightly higher than their E-face association (Wolburg et al., 1994) (Figure 1-4c). The P-face/E-face ratio of BBB tight junctions continuously increases during development (Kniesel et al., 1996). In cell culture, where the transendothelial resistance is much lower as in vivo, the freeze-fracture morphology of BBB endothelial cells is similar to non-BBB endothelial cells (Figure 1-4d) indicating that the association of the strand particles with the membrane leaflets reflects the quality of the barrier and is under the control of the brain microenvironment (Wolburg et al., 1994; Liebnev et al., 2000a).

5 The Molecular Composition of Tight Junctions

In the last 10 years, the knowledge of the molecular composition and regulation of the tight junctions has rapidly extended (Furuse et al., 1993, 1998; Ando-Akatsuka et al., 1996; Morita et al., 1999a; Tsukita et al., 1999, 2001; Heiskala et al., 2001; Huber et al., 2001; D’Atri and Citi, 2002; Gonzalez-Mariscal et al., 2003; Matter and Balda, 2003; Dejana, 2004; Turksen and Troy, 2004) (Figure 1-5, Table 1-1). Occludin and the claudin family are the most important membranous components both of which are proteins with four transmembrane domains and two extracellular loops.

5.1 Occludin

Occludin was the first tight junctional transmembrane molecule discovered (Furuse et al., 1993). It was initially isolated from junction-enriched membrane fractions of the chicken liver as a transmembranous tight junction protein of ~65 kDa, which exists in several isoforms. Occludin shows high interspecies variability between chicken and mammals (Ando-Akatsuka et al., 1996), sharing less than 50% identity in amino acid sequence. In contrast, human, murine, and canine occludins are more closely related, showing ~90% identity. Besides the high content of tyrosine and glycine in the first extracellular loop (~60%), the most conserved region of occludin comprises the carboxy terminal ZO-1 binding domain, an α-helical coiled coil structure, putatively linking occludin to the cytoskeleton.

Surprisingly, the tight junctions in occludin-deficient mice (Saitou et al., 2000) were not affected morphologically, and transepithelial resistance as measured in small and large intestine epithelial cells was not altered compared to wild-type mice. However, the mice developed chronic inflammation and...
Table 1-1
Molecular composition of tight junctions

<table>
<thead>
<tr>
<th>Integral membrane proteins</th>
<th>Adaptor proteins first order (direct)</th>
<th>Adaptor proteins second order (indirect)</th>
<th>Signaling proteins</th>
<th>Regulatory proteins</th>
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<tr>
<td><strong>Tight junction strand proteins</strong></td>
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<tr>
<td>Occludin</td>
<td>ZO-1, -2, -3</td>
<td>AF-6, cingulin</td>
<td>PI3-kinase, CK2, PKC</td>
<td>Itch</td>
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<tr>
<td>Claudins (CI-3, -5, -12)</td>
<td>ZO-1</td>
<td>AF-6, cingulin</td>
<td>c-Yes, Gα 12</td>
<td>ZONAB</td>
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<td></td>
<td>ZO-2</td>
<td>cingulin</td>
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<td>c-Jun, c-Fos, C-EBP, SAF-B</td>
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<td></td>
<td>ZO-3</td>
<td>PATJ, cingulin</td>
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<td></td>
<td>MUPP1</td>
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<td><strong>Ig-superfamily members</strong></td>
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<tr>
<td>JAM-A</td>
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<td>PAR-6</td>
<td>aPKC, PP2A</td>
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<td>JAM-C</td>
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<td>PAR-6</td>
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<td>CLMP</td>
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<td><strong>Crumb homologs</strong></td>
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<td>aPKC, PP2A</td>
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The molecular components identified at tight junctions are grouped into different classes based on their structures and functions. The first column displays integral membrane proteins. The second column displays adaptor proteins and distinguishes between first- and second-order adaptors based on their direct or indirect association with the integral membrane proteins. The third column contains signaling proteins, which include tyrosine and serine/threonine kinases as well as heterotrimeric G-proteins, small GTP-binding proteins, and guanine-nucleotide exchange factors. Molecules in the fourth column include regulatory proteins such as transcription factors, transcription regulatory proteins, or proteins regulating posttranslational modifications. The inclusion of the molecules listed in the third and fourth column in the table is based on either their association with adaptor proteins or their localization at tight junctions. The mere association with a tight junction associated protein does not necessarily mean that the two proteins are associated at tight junctions. Actin-binding proteins,
hyperplasia of the gastric epithelium, calcifications in the brain and around brain vessels, thinning of bones, postnatal growth retardation, testicular atrophy, and abnormalities in sexual behavior (Saitou et al., 2000). The authors concluded that occludin might have a function in tight junction modulation via the induction of intracellular signaling. Moreover, occludin is not required for the formation of tight junction strands. In a number of reports, posttranslational modifications of occludin, such as phosphorylation of its cytoplasmic domains or binding to a ubiquitin-ligase (Traweger et al., 2002), have been described as parts of tight junction regulation (Chen et al., 1997; Sakakibara et al., 1997; Balda et al., 2000; Huber et al., 2000; Hirase et al., 2001). For example, DeMaio et al. (2001) reported on a clear reduction of occludin content in cultured aortic endothelial cells by shear stress (10 dyn/cm²) but a time-dependent increase of occludin phosphorylation, which could be attenuated by dibutyryl cAMP. An increase of occludin phosphorylation has also been described after treatment with VEGF (Antonetti et al., 1999), suggesting that hormonal and mechanical changes are able to increase the paracellular permeability by an early increase of occludin phosphorylation and a subsequent decrease of the occludin content. Taken together, it seems that mature cells need occludin to regulate rather than establish their barrier properties.

5.2 The Claudin Family

The claudins are the tight junction molecules that seem to fulfill the task of establishing barrier properties (Furuse et al., 1999, 2001; Morita et al., 1999a; Turksen and Troy, 2004) (Table 1.1). Claudins share with occludin the overall organization with four transmembrane domains but have no sequence homology to occludin. The first claudins identified were isolated from chicken liver junctional fractions and were called claudin-1 and -2 (Furuse et al., 1998). Since then a number of related proteins have been identified and at present the claudin family contains more than 20 members (Mitic et al., 2000). It is now believed that claudins are responsible for the regulation of paracellular permeability through the formation of homotypic and heterotypic paired strands (for a review, see Tsukita et al., 2001; Turksen and Troy, 2004). In this model, ion selectivity is achieved through the selective expression and combination of distinct claudins in certain tissues (Tsukita and Furuse, 2000). Therefore, it is not surprising that the claudins are not randomly distributed throughout the organs but, at least in part show a tissue-specific expression pattern. For example, claudin-5 was originally described to be restricted to endothelial cells (Morita et al., 1999b), although it was later found in surface cells of the stomach and of the large and small intestine also (Rahner et al., 2001). In addition, claudin-16 is selectively expressed in the thick ascending limb of Henle in the kidney, where it regulates selectively the permeability for Mg\textsuperscript{2+} ions (Simon et al., 1999).

Functional investigations support the view that the composition of the claudin species directly determines barrier function (Furuse et al., 2001). Tight junction–negative L-fibroblasts when transfected with claudin-1 or -3 form tight junctions, which appeared in freeze-fracture replicas associated with the P-face (Furuse et al., 1999). When transfected with claudin-2 or -5, the cells form tight junctions associated with the E-face (Furuse et al., 1998; Morita et al., 1999b). In contrast, occludin was found to be localized at both fracture faces (Hirase et al., 1997). Whereas occludin induced the formation of short strands, the claudin-induced strands were very long and branched resembling endogeneous tight junctions (Tsukita and Furuse, 1999; Furuse et al., 1999).
Tight junctions in the blood–brain barrier

Under pathological conditions, such as malignant glioma or experimental allergic encephalomyelitis (EAE), tight junctions that are associated with the E-face associated (Wolburg et al., 1994) and express less claudin-1 (Liebner et al., 2000a, b); however, an antibody was used that is now known to recognize claudin-3 as well. Under pathological conditions, such as malignant glioma or experimental allergic encephalomyelitis (EAE), claudin-1 or -3 was found to be lost and/or the tight junctions were E-face associated (Wolburg et al., 2003).

5.3 Immunoglobulin-like Proteins at Tight Junctions

Almost simultaneously with the identification of claudins, junctional adhesion molecule (JAM) has been reported as the first member of the immunoglobulin (Ig) superfamily to be present at tight junctions.
(Martin-Padura et al., 1998) (Figure 1-5, Table 1-1). JAM, which is now called JAM-A, localizes at homotypic cell–cell contacts of endothelial and epithelial cells and is highly enriched at tight junctions (Martin-Padura et al., 1998; Liu et al., 2000). Two Ig-like proteins closely related to JAM-A, JAM-B, and JAM-C have been identified (Palmeri et al., 2000; Aurrand-Lions et al., 2000). JAM-B and JAM-C are restricted to endothelial cells and are largely absent from epithelial cells. Although ultrastructural analyses for JAM-B and JAM-C in endothelial cell are still missing, the colocalization of JAM-C with occludin and ZO-1 (see later) upon ectopic expression in MDCK epithelial cells suggests its localization at tight junctions (Aurrand-Lions et al., 2001). Additional evidence for a role of JAMs in the formation of tight junctions is based on the observation that anti-JAM-A antibodies as well as soluble JAM-A negatively affect the formation of functional tight junctions after Ca²⁺-switch–induced cell–cell contact formation (Liang et al., 2000; Liu et al., 2000) and the identification of cytosolic proteins which associate with JAMs and are implicated in the formation/function of tight junctions (see later). In this respect, it is interesting to note that JAM-A and JAM-B are expressed by Sertoli cells in the testis, where they could be involved in the formation and/or maintenance of the blood–testis barrier (Gliki et al., 2004).

Another role of JAMs in endothelial cells might be related to their predicted function in regulating leukocyte–endothelial cell interaction during inflammation through homophilic and heterophilic interactions (for recent reviews, see Johnson-Léger and Imhof, 2003; Muller, 2003; Ebnet et al., 2004). Originally, blocking JAM-A was found to inhibit leukocyte diapedesis in vitro and during inflammation in vivo (Del Maschio et al., 1999). However, there is a considerable body of evidence that leukocyte diapedesis must not follow the paracellular route but the transcellular route via a mechanism called emperiploisis (for overviews of both the classical transmigration studies and the recent literature, see Carman and Springer, 2004; Engelhardt and Wolburg, 2004). The transcellular mechanism as observed in EAE leaves tight junctions intact and implies a complex rearrangement of the luminal and abluminal membranes (Wolburg et al., 2005). This does not exclude that despite leaving tight junctions morphologically intact during transcellular transmigration, these junctions can molecularly be changed. In EAE, we demonstrated a selective loss of anti-claudin-3 immunoreactivity (Wolburg et al., 2003). This molecular alteration is associated with an increase in vascular permeability but not an opening of tight junctions for paracellular leukocyte diapedesis. If we, therefore, assume that transcellular migration of leukocytes delivers a signal to the endothelial junction—probably via the actin cytoskeleton—it is tempting to speculate that functional antibodies that “tickle” junctional molecules, such as VE-cadherin, PECAM-1, the JAM family, or CD99, might trigger these intracellular signaling cascades so that they increase or decrease endothelial mechanisms required for either transcellular or paracellular migration.

Four additional Ig-superfamily members have been identified at tight junctions (Table 1-1). These include the coxsackie- and adenovirus receptor (CAR) (Cohen et al., 2001), endothelial cell-selective adhesion molecule (ESAM) (Nasdala et al., 2002), JAM-4 (Hirabayashi et al., 2003), and coxsackie- and adenovirus receptor-like membrane protein (CLMP) (Raschperger et al., 2004). They share with JAM-A, JAM-B, and JAM-C—a similar organization with two Ig-like domains. However, they are more closely related with each other than to the three JAMs and thus form a subfamily within tight junction–associated Ig-superfamily members (Ebnet et al., 2004). CAR, ESAM, and JAM-4 end in a type I PDZ domain-binding motif whereas JAM-A, JAM-B, and JAM-C end in a type II motif, which suggests functional differences between the two subfamilies. The function of these four Ig-superfamily members at tight junctions is not clear. CAR, JAM-4, and CLMP are predominantly expressed by epithelial cells, whereas ESAM is expressed exclusively in endothelial cells including those in brain capillaries (Hirata et al., 2001; Nasdala et al., 2002). Endothelial cells derived from ESAM-deficient mice display defects in endothelial tube formation suggesting a role for ESAM in endothelial cell contact formation (Ishida et al., 2003). How this function relates to its specific localization at tight junctions is not yet clear.

5.4 Peripheral Membrane Components at Tight Junctions

The transmembrane proteins associate in the cytoplasm with peripheral membrane components, which form large protein complexes, the cytoplasmic “plaque” (Figure 1-5, Table 1-1). ZO-1, a 220-kDa
phosphoprotein, was the first peripheral membrane component identified and characterized at tight junctions (Stevenson et al., 1986). In cellular systems with less elaborate or no tight junctions at all, ZO-1 is found enriched in regions of the adherens junctions (Itoh et al., 1993), where it may interact with components of the cadherin–catenin system (Rajasekaran et al., 1996; Itoh et al., 1997). Since the discovery of ZO-1, many further components of peripheral tight junction proteins have been described. One type of plaque proteins consists of adaptors, proteins with multiple protein–protein interaction domains such as SH-3, guanylate kinase (GUK), and PDZ domains (Vohno, 2001; Harris and Lim, 2001; Pawson and Nash, 2003). The adaptor proteins include members of the membrane-associated guanylate kinase (MAGUK) (Anderson, 1996) and membrane-associated GUK with an inverted orientation of protein–protein interaction domains (MAGI) (Dobrosotskaya et al., 1997) families, such as ZO-1, -2, -3, Pals-1, MAGI-1, -2, and -3, as well as proteins with one or several PDZ domains such as PAR-3, PAR-6, PATJ, and MUPPI (Ebnet et al., 2001; Ohnno, 2001; Hamazaki et al., 2002; Ebnet et al., 2004). The latter two proteins contain 10 and 13 PDZ domains (Ullmer et al., 1998; Roh et al., 2002), respectively, and thus seem to be particularly well suited to assemble large protein complexes. The adaptor proteins serve as scaffolds to organize the close proximity of the second type of plaque proteins, the regulatory and signaling proteins. These include small GTPases like Ras, Rab13, or Cdc42 (Hopkins et al., 2000), and their regulators, e.g., guanine nucleotide exchange factors (Benais-Pont et al., 2003), protein kinases, and phosphatases, such as atypical protein kinase C (aPKC), PP2A, and PTEN (Izumi et al., 1998; Wu et al., 2000; Avila-Flores et al., 2001; Nunbhakdi-Craig et al., 2002), as well as transcriptional regulators like ZO-1-associated nucleic acid-binding protein (ZONAB), huASH1, Jun, Fos, and the CCAAT/enhancer binding protein (C/EBP) (Nakamura et al., 2000; Balda et al., 2003; Betanzos et al., 2004). Furthermore, a new protein called JACOP (junction-associated coiled-coil protein) has been discovered. This protein has been found in the tight junction complex of epithelial and also endothelial cells and is suggested to anchor, especially, the junctional complex to the actin-based cytoskeleton (Ohnishi et al., 2004). JACOP has considerable sequence similarity to cingulin, another previously detected peripheral protein at tight junctions (Citi et al., 1989). In many cases, the role of the regulatory and signaling proteins in tight junction biology is still poorly understood, and it is expected that they are involved in completely different aspects of tight junction biology. The proteins of the PAR-3–aPKC–PAR-6 complex are most likely involved in the regulation of tight junction formation and establishment of cell polarity since overexpression of dominant-negative mutants of these proteins lead to a delayed tight junction formation (Suzuki et al., 2001; Gao et al., 2002; Nagai-Tamai et al., 2002). In addition, when dominant-negative mutants of the PAR-3 ligand JAM-A are overexpressed in epithelial cells, this leads to a delay in tight junction formation and to defects in the establishment of cell polarity (Rehder D and Ebnet K, unpublished results 1918. Both JAM-A and PAR-3 localize to cell–cell contacts of endothelial cells (Ebnet et al., 2003), and therefore it is conceivable that the formation of tight junctions is similarly regulated by JAM-A and the PAR-3–aPKC–PAR-6 complex. Other proteins present at tight junctions might be necessary for signaling to the cell interior, for example, the transcription factors ZONAB and huAsh1. Information on the maturation state of cell–cell contacts is required for many cellular events, which are regulated by cell density (e.g., proliferation), and transcription factors associated with the cytoplasmic plaque at tight junction provide a direct link between tight junctions and the nucleus (Matter and Balda, 2003).

The vast majority of experiments addressing the role of tight junction–associated proteins for tight junction biology were performed with epithelial cells. Our knowledge about their role in tight junction formation in endothelial cells, and in particular those in the BBB, is still limited. However, it is expected that the principal mechanisms underlying tight junction formation operate in both cellular systems in a similar way.

6 Regulatory Mechanisms in the Blood–Brain Barrier

Tight junctions have long been considered as static barriers responsible for both the compartmentalization of the intercellular cleft (gate function) and the polarity of cell (fence function). But in the last years, it came out that the tight junctions are under the control of multiple regulatory systems in which many molecular systems, such as adhesion molecules, extracellular matrix components, and signal transduction pathways,
are involved (Matter and Balda, 2003; Sheth et al., 2003). Most data were gathered in epithelial cells. In endothelial cells, in particular in those of the BBB, less data are available, probably due to the fact that the system is considerably more complex as it includes pericytes and astrocytes, a special composition of the extracellular matrix, and not well established in vitro.

6.1 G-Protein Signaling

G-proteins play an essential role in maintaining barrier integrity of epithelial cells (Matter and Balda, 2003). The G-proteins involved are the classical heterotrimeric G-proteins and the small G-proteins/small GTPases (Ras superfamily). Several Gα subunits have been localized within the tight junctions of cultured epithelial cells, such as Gα₁₂ (Denker et al., 1996), Gαs (Dodane and Kachar, 1996), Gα₁₂ (Dodane and Kachar, 1996), and Gα₁₅, when transfected into MDCK cells (Nakamura et al., 2000). Moreover, Gα₁₂ (Denker et al., 1996) and Gα₁₂ (Meyer et al., 2002) could be coprecipitated with ZO-1. The activation of heterotrimeric G-proteins leads to activation of second messengers like cAMP/cGMP or Ca²⁺ and to an increased transepithelial resistance of the cell monolayer. Additionally, Gα₁₂ accelerated tight junction biogenesis, whereas Gα₁₅ was important for development and maintenance of the tight junctions (Denker and Nigam, 1998; Saha et al., 2001). Gα₁₂, in contrast, has been demonstrated to be involved in the tight junction maintenance in part through Src tyrosine kinase pathways. A constitutively active Gα₁₂ disrupted adherens and tight junctions and increased actin stress fibers leading to increased paracellular permeability in MDCK cells (Meyer et al., 2003). The inhibition of the protein kinase A (PKA) has been shown to result in the preservation of tight junctions and low permeability in MDCK cells during removal of calcium (Klingler et al., 2000), suggesting that in epithelial cells the PKA could be involved in the destabilization of tight junctions (Balda et al., 1991). As activated Gα₁₂ evokes a decrease in cAMP and thus probably a decrease in the amount of activated PKA, G-protein signaling could influence epithelial permeability via inhibiting the cAMP/PKA-pathway. In contrast, in endothelial cells, elevation of cAMP by forskolin resulted in a stabilization of tight junctions and decrease of permeability (Rubin et al., 1991; Wolburg et al., 1994; Raub, 1996). Accordingly, elevation of cAMP by forskolin or cholera toxin was able to reverse the permeability increasing effect of pertussis toxin (PTX) on cerebral endothelial cells in vitro (Brückener et al., 2003). PTX is known to inhibit G-protein signaling by ADP-ribosylation of Gα_i proteins. The pathway by which PTX permeabilized the barrier was shown to include the PKC (Brückener et al., 2003), probably by operating via extracellular signal-regulated kinase (ERK) activation (Garcia et al., 2001).

A family of G-protein signaling regulating proteins has been identified. These so-called RGS proteins (regulators of G-protein signaling) interact with the Gα subunit of heterotrimeric G-proteins (Figure 1-5). They inactivate the Gα subunit by accelerating GTP hydrolysis (De Vries and Farquahr, 1999). A genomic suppression subtractive hybridization (SSH) approach has been used to identify specific genes expressed at the BBB (Li et al., 2001a, b). Among other genes, RGS5 was found to be expressed at the BBB and also in other tissues. By using the same approach in hypertensive rats, the RGS5 mRNA was identified in isolated brain capillaries and localized in the BBB endothelial cells (Kirsch et al., 2001) (Figure 1-5). Immunocytochemical detection of RGS5 at the light and electron microscope level revealed its occurrence in tight junctions of BBB endothelial cells (Lippoldt et al., in preparation). In an in vitro model of capillary morphogenesis using human umbilical vein endothelial cells (HUVECs), RGS5 was mostly downregulated during the time of extensive branching (Bell et al., 2001) pointing to its importance in endothelial cell differentiation. However, the function of RGS5 in endothelial cells or the signaling pathway RGS5 is acting on is not yet clear. The G-protein connected receptor as marked in Figure 1-5 as “Rec” is postulated but not found so far.

6.2 Small GTPases

The RhoA and Rac1 small GTPases were shown to play a promoting role in the regulation of tight and adhesion junction structure and function (Adamson et al., 2002; Van Hinsbergh et al., 2002; Matter and Balda, 2003) (Figure 1-5). In MDCK-cells expressing RhoA and Rac1 mutants, the organization of tight
junctions is disturbed and the permeability for inulin, anionic or neutral dextran increased and the TER decreased (Jou and Nelson, 1998; Jou et al., 1998). At the molecular level, the GTPases RhoA, Rac1, and Cdc42 promoted the internalization of the tight junction transmembrane molecules occludin, claudin-1 and -2, JAM-1, and ZO-1 depending on their activation status and in a GTPase specific manner (Bruewer et al., 2004). In T84 cells, the inhibition of the Rho pathway by Clostridium botulinum toxin C3 transferase resulted in a disorganization of the perijunctional actin ring and ZO-1 distribution, whereas the transient expression of RhoC resulted in actin concentration at intercellular contacts (Nusrat et al., 1995). On the other hand, Escherichia coli cytotoxic necrotizing factor-1 (CNF-1) activated the Rho pathway but reduced the gate function of tight junctions in T84 cells and impaired tight junction assembly in the calcium switch assay (Hopkins et al., 2002) by occludin and caveolin-1 internalization in endosomal-caveolar–like structures and ZO-1 and JAM-1 displacement from the tight junctions (Hopkins et al., 2003). In addition, a guanine nucleotide exchange factor (GEF) has been identified, which activated Rho associated with tight junctions and increased the paracellular permeability in MDCK cells (Benais-Pont et al., 2003). Regarding cerebral endothelial cells, the activation of the Rho pathway in vitro by lysophosphatidic acid (LPA) (van Leeuwen et al., 2003) disrupted the paracellular barrier (Schulze et al., 1997). Accordingly, the inhibition of the Rho pathway prevented the LPA-induced increase in permeability (Balda et al., 2000). Inflammation, as caused by bradykinin, thrombin, histamin cytokines, matrix metalloproteinases (Lum and Malik, 1996; Wöjciak-Stothard et al., 1998; Johnson-Léger et al., 2000; Mayhan, 2001; Liu et al., 2001; Petty and Lo, 2002), or by bacterial toxins (Essler et al., 1998, 2000; Adamson et al., 1999, 2002) increases the transendothelial permeability by affecting tight and adherens junctions via reorganization of the actin cytoskeleton and formation of intercellular gaps. Also the transendothelial migration of lymphocytes was shown to be dependent on Rho signaling—when activation of Rho as a consequence of leukocyte binding to the adhesion receptor ICAM-1 was blocked by C3-transferase the leukocyte transmigration was compromised (Adamson et al., 1999). Whereas the extracellular domain of endothelial ICAM-1 suffices to mediate T cell adhesion, the cytoplasmic domain is required to mediate transmigration of T cells probably by inducing Rho-signaling within the endothelial cells (Etienne et al., 1998). In HUVECs, affection of the permeability requires the activation of the Cdc42-, rac-, Rho-cascade following stimulation by TNF-α (Lum and Malik, 1996). Furthermore, in vitro studies demonstrated that a Rho/Rho kinase-dependent pathway is a central target for an increase of vascular permeability by inactivating the myosin light chain (MLC) phosphatase, thus enhancing MLC phosphorylation. This leads to endothelial cell contraction and increased permeability (Essler et al., 2000).

**7 Conclusions**

The neurogliovascular complex is involved in the regulation of blood flow and nutrient supply within the CNS. This regulation includes: (1) control of perfusion parameters differentially realized in specific brain regions according to local requirements, (2) maintenance of energy supply from blood to neuronal and synaptic metabolism via glial cells, and (3) the protection of the nervous parenchyma from alterations of blood composition, in particular, from neurotoxic compounds including reactive oxygen species. Although the cellular partners of the neurogliovascular complex, such as neurons, astrocytes, pericytes, and endothelial cells, are well-known since many years, a functional understanding of what and to which end is signaled between these cells in order to establish the blood–brain interface is far away from being complete. The most important advance in the last years in this field seems to be the recognition of the fact that this interface is not simply an attribute of the brain microvessels but the result of an intense active interaction of all cellular and molecular partners during brain development and barrier maintenance. The formation and continuous maintainance of tight junctions between BBB endothelial cells is only the last step in a long chain of processes characterized now as the common effort of the brain microenvironmental factors to realize energy supply, control of perfusion, and protection from the blood.

Tight junctions have been primarily described as a network of protein particles using freeze-fracture electron microscopy. But, it needed almost 30 years and a great methodological advance in molecular biology to discover protein components of the tight junctional complex and to identify the protein particles
within the replicas. Moreover, due to the advances in molecular biological methods, like knockout and GFP-technologies, it is now possible to recognize the tight junctional complex as a very dynamic structure. Using these approaches (Sasaki et al., 2003), it turned out that paired claudin strands are the molecular equivalents of the so-called “kissing points” seen in transmission electron microscopy. In vitro time lapse studies of annealing tight junction strands possessing different types of claudins for the first time demonstrated the high dynamic organization potential of the tight junctional complex. Now, it might be possible to go more into detail to characterize the interplay of these molecules and their regulation by and association with cellular signaling cascades and cytoskeletal components. These data then will allow us to use this knowledge for therapeutic interventions in pathologies influencing BBB integrity.

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