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Review

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Physiology of Blood–Brain Interfaces in Relation to Brain Disposition of Small Compounds and Macromolecules

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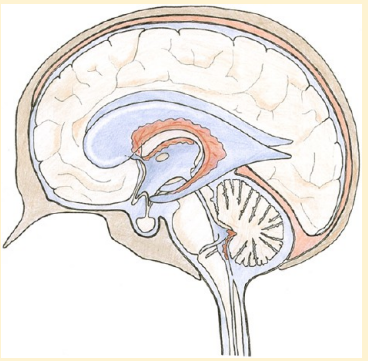
Supporting Information

ABSTRACT: The brain develops and functions within a strictly controlled environment resulting from the coordinated action of different cellular interfaces located between the blood and the extracellular fluids of the brain, which include the interstitial fluid and the cerebrospinal fluid (CSF). As a correlate, the delivery of pharmacologically active molecules and especially macromolecules to the brain is challenged by the barrier properties of these interfaces. Blood–brain interfaces comprise both the blood–brain barrier located at the endothelium of the brain microvessels and the blood–CSF barrier located at the epithelium of the choroid plexuses. Although both barriers develop extensive surface areas of exchange between the blood and the neuropil or the CSF, the molecular fluxes across these interfaces are tightly regulated. Cerebral microvessels acquire a barrier phenotype early during cerebral vasculogenesis under the influence of the Wnt/β-catenin pathway, and of recruited pericytes. Later in development, astrocytes also play a role in blood–brain barrier maintenance. The tight choroid plexus epithelium develops very early during embryogenesis. It is specified by various signaling molecules from the embryonic dorsal midline, such as bone morphogenic proteins, and grows under the influence of Sonic hedgehog protein. Tight junctions at each barrier comprise a distinctive set of claudins from the pore-forming and tightening categories that determine their respective paracellular barrier characteristics. Vesicular traffic is limited in the cerebral endothelium and abundant in the choroidal epithelium, yet without evidence of active fluid phase transcytosis. Inorganic ion transport is highly regulated across the barriers. Small organic compounds such as nutrients, micronutrients and hormones are transported into the brain by specific solute carriers. Other bioactive metabolites, lipophilic toxic xenobiotics or pharmacological agents are restrained from accumulating in the brain by several ATP-binding cassette efflux transporters, multispecific solute carriers, and detoxifying enzymes. These various molecular effectors differently distribute between the two barriers. Receptor-mediated endocytotic and transcytotic mechanisms are active in the barriers. They enable brain penetration of selected polypeptides and proteins, or inversely macromolecule efflux as it is the case for immnoglobulins G. An additional mechanism specific to the BCSFB mediates the transport of selected plasma proteins from blood into CSF in the developing brain. All these mechanisms could be explored and manipulated to improve macromolecule delivery to the brain.



KEYWORDS: *blood–brain barrier, cerebrospinal fluid, choroid plexus, development, immunoglobulin, pinocytosis, receptor mediated transcytosis, tight junctions, transporter*

1. INTRODUCTION

Delivery of pharmacologically active molecules, especially macromolecules, to the brain is challenged by specific properties of the cells forming the interfaces between the blood and the central nervous system (CNS). By restricting both paracellular and transcellular diffusion of hydrophilic and lipophilic substances, these cellular interfaces provide the strictly controlled environment required for CNS development and functions. They are collectively named blood–brain interfaces and are present in various locations as illustrated in Figure 1. The barrier between the blood and the brain or spinal cord parenchyma proper, hereafter referred to as the blood–brain barrier (BBB), is formed by the endothelium of the cerebral microvessel (Figures 1 and 2A). This barrier develops a large surface area of exchange between the blood and the neuropil, with an average of 100 cm^2 per gram of brain tissue in adult mammal.[1] All capillaries of gray and white matter structures including those of the hypothalamus display a tight barrier phenotype. By contrast, the capillaries of circumventricular organs such as the area postrema or the median eminence are highly permeable and form the exception among brain structures. The presence of permeable capillaries in these latter structures is relevant to their functions which include sensing of blood-borne hormones and other products, and central control of diverse homeostatic processes.[2] Circum-

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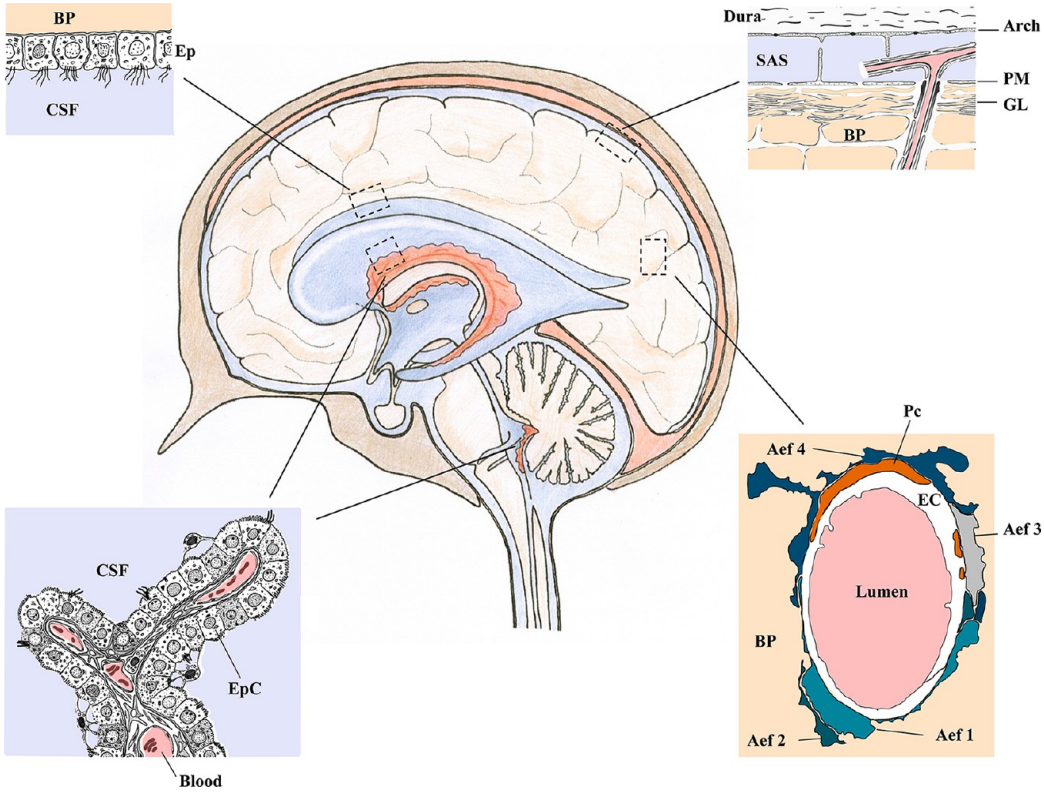

Figure 1. Cellular interfaces between blood, CSF, and brain parenchyma. The CSF-filled spaces (in blue) and the major different brain interfaces are illustrated on the schematic representation of a human brain. Insets are as follows (clockwise from lower right). The BBB: the endothelial cells and the closely apposed pericyte are completely unsheathing by overlapping astrocytic end-feet (schematized from an electron micrograph in ref 12). The BCSFB: the polarized epithelial cells contacting the CSF delimit a highly vascularized inner stromal core. Choroidal vessels are fenestrated. The ependyma: ependymal cells form the interface between brain and ventricular CSF. The arachnoid interface: the arachnoid membrane separates the CSF-containing subarachnoid spaces from the dura. Penetrating vessels cross the *pia mater* and *glia limitans* at the surface of the brain facing the subarachnoid, or cisternal (not shown here) CSF spaces. Tight junctions are present between endothelial cells of parenchymal microvessels, epithelial cells of the choroidal epithelium, cells of the arachnoid membrane and endothelial cells of pial vessels. They are absent in the ependyma, *pia mater*, and *glia limitans*, allowing paracellular diffusion between CSF and brain. Abbreviations: Aef, astrocytic end-foot; Arach, arachnoid membrane; CSF, cerebrospinal fluid; EC, endothelial cell; Ep, ependyma; GL, *glia limitans*; EpC, epithelial cell; *Pc*, pericyte; PM, *pia mater*; SAS, subarachnoid space.

ventricular organs, however, do not constitute a significant route of entry into the whole CNS for blood-borne pharmacological compounds. The surface area developed by the endothelium of the penetrating cerebral arteries and veins is also modest by comparison to the surface developed by the microvessel network. Furthermore, the endothelium of these vessels shares several of the barrier properties of the capillary endothelium. The BBB is to be distinguished from a second barrier component, located between the blood and the ventricular cerebrospinal fluid (CSF), and called the blood–CSF barrier (BCSFB). The latter interface is formed by the tight epithelium of the choroid plexuses, which are specialized structures projecting in all four ventricles of the brain and are responsible for the active secretion of CSF (Figures 1 and 2B). This barrier also develops a substantial surface area of exchange which is certainly more important than previously suggested, owing to complex interdigitations of the basolateral membrane of choroidal epithelial cells and a dense network of microvilli at their apical membrane. In a one-month-old rat, the apical surface area in contact with CSF has been estimated to be 75 cm^2, i.e., close to the surface area developed by the BBB.[3] In addition to this large surface area, the choroid plexuses possess the highest local blood flow rate among all cerebral structures, and thereby contribute to a significant extent to the exchanges between blood and CNS. A third tight interface separates the subarachnoid CSF from the bones and *dura mater* extracellular fluids. It stands at the level of the arachnoid membrane which totally encircles the CNS and is closely apposed to the *dura mater* (Figure 1).

The CSF system fulfills several functions which range from buoyancy, drainage, and buffering to volume transmission, neuroendocrine signaling, and neuroimmune surveillance and is being implicated in an increasing number of associated pathologies. The flow of CSF, brisk in the ventricular system, slower in the subarachnoid and cisternal compartments, allows both the drainage of the brain and the delivery of active molecules secreted in or transported by the CSF to remote brain areas. The latter areas include fluid-filled subarachnoid and cisternal spaces, velae, and perivascular spaces, brain regions close to CSF such as periventricular and pericisternal areas, and, for continuously secreted factors, deeper brain tissue (reviewed in refs 4–6). The fluid is eventually resorbed by arachnoid projections in venous sinuses, and/or along cranial and spinal nerve roots in extracranial lymphatics.[7,8]

Within the CNS, exchanges between the CSF and the parenchyma occur for one part across the ependymal layer lining the ventricular system (Figures 1 and 3A). In most places, the ependyma is formed by a single layer of cuboidal

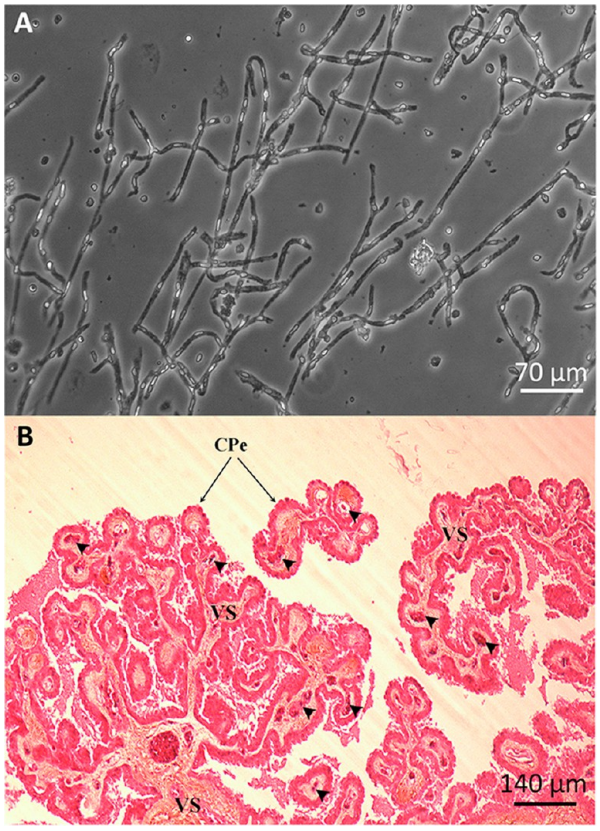

Figure 2. Anatomical basis of the blood–brain interfaces. (A) The BBB lies at the level of the endothelium of cerebral capillaries and microvessels. Capillaries can be isolated owing to the continuous basal membrane surrounding the endothelial cells and pericytes. Capillaries isolated from adult rat are shown in this phase-contrast micrograph. Occasional red blood cells are still visible in the lumen. (B) The BCSFB lies at the level of the choroidal epithelium (CPe), which organizes in numerous villi and surrounds a conjunctive highly vascularized stroma (VS). The histological section through an adult human choroid plexus (paraffin-embedded tissue, hemalum phloxine saffron staining) illustrates the numerous epithelial villi that enhance the surface area of the barrier, and the extensive vascular network (some vessels indicated by arrowheads).

cells without tight junctions, thus exerting no restriction on the paracellular diffusion of hydrophilic molecules.[9] Exchanges also occur in a more complex mode between the subarachnoid and cisternal CSF and the underlying neuropil, across the permeable *pia mater* and *glia limitans* lying immediately underneath. The pial membrane does not hinder the movement of molecules between the subarachnoid CSF and the brain, but the *glia limitans* (Figures 1 and 3B), which in most places forms a complex network of glial cell membranes, may either slow down diffusion or else be a site of accumulation for some compounds.[4]

There are two fundamental components contributing to the barrier phenotype of the blood–brain cellular interfaces. The first one is structural, and depends on the existence of continuous tight junctions between adjacent cells in the endothelium, the choroidal epithelium, and the arachnoid membrane. Tight junctions greatly limit the exchanges of hydrophilic molecules between blood and brain or CSF. The second component is sustained by a large number of transport systems on both cell membranes, as well as intracellular metabolic activities. Inward transport mechanisms provide the

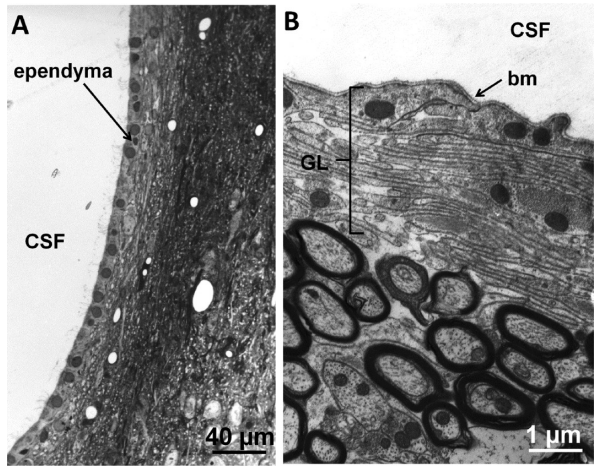

Figure 3. Anatomical basis of the brain–CSF interfaces. (A) The ependyma that lines the ventricles of the brain is in most places formed by a simple epithelium-like monolayer (semithin 1 μm thick section of rat brain). Ependymal cilia can be distinguished. Open white areas in the tissue are microvessel lumens. (B) The neuropil facing the pial membrane and subarachnoid/cisternal CSF is bordered by the *glia limitans* (GL) lying on a basal membrane (bm). The *glia limitans* displays several layers of intermingled plasma membrane. Electron microscopy of the ambient cistern border is shown. Myelinated nerve fibers are seen in the neuropil.

brain with all nutrients, micronutrients and hormones required for cerebral functions and homeostasis. The transcellular transport systems also include a number of outward transporters which, in conjunction with detoxifying enzymes, impede the penetration of harmful compounds in the brain or favor their elimination from the brain. These systems also recognize pharmacological agents developed for CNS therapy, and can reduce their cerebral bioavailability and efficacy.

This review will describe the basic anatomy and genesis of the blood–brain interfaces, and present the distinctive attributes responsible for their barrier phenotype. These encompass the structural features that physically restrict the paracellular pathway, i.e., the tight junctions, and the inward and outward transport systems that regulate transcellular fluxes between blood and brain. The specificities distinguishing the BBB from the BCSFB will be underlined. The last section will then explore in more detail some of the putative transcytotic mechanisms influencing the delivery of hydrophilic macromolecules (peptides and proteins) to the brain, focusing on the BCSFB.

2. BASIC ANATOMY AND GENESIS OF THE BARRIERS

2.1. The Blood–Brain Barrier. The term "blood–brain barrier" was introduced at the turn of the 20th century to define the existence of barrier mechanisms impeding the exchanges of dyes between the brain and the body.[10] With the characterization of these mechanisms and a clearer understanding of their regulation, the concept of "neurovascular unit" has been recently introduced.[11] It integrates the multiplicity of cells that associate and communicate to form a functional barrier between the blood and the interstitial fluid of the brain. The neurovascular unit comprises the microvascular endothelial cell with its basal lamina unsheathing closely apposed pericytes, astrocytic end-feet with their own basement membrane covering the vessel wall, and occasional microglial cells and neuronal terminals (Figure 1, inset illustrating a schematic cross

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sectional representation of a typical cerebral microvessel, modified from a 3-dimensional reconstruction study). A recent 3-dimensional reconstruction study in the rat hippocampal CA1 layer has provided new insights on how all of these cells spatially organize within the neurovascular unit, at least in this particular brain region.[12] It confirmed that the glial sheath provides an almost complete coverage of the brain microvessel wall, with notable substantial overlaps of adjacent end-foot processes. Only in limited areas was the coverage interrupted. This occurred at the level of certain pericytes, thus permitting direct contact of the brain-facing surface of these cells with elements of the neuropil. Other discontinuities in the perivascular astrocytic end-feet existing over the endothelial basement membrane were filled with cellular processes thought to be of microglial origin. Finally, perivascular nerve terminals have also been described to interrupt the astrocytic ensheathment.[13]

The cross talk between the different cellular constituents of the neurovascular unit has been studied for the last couple of decades. Studies focused first on the interactions between endothelial cells and astrocytes and were mostly performed using in vitro cellular systems. These works provided evidence of astrocyte-secreted soluble factors, which are able to reinduce certain transporter-mediated properties in the cultured endothelial monolayers and to decrease their permeability to small polar compounds (reviewed in ref 14). Yet, the influence of astrocytes, which develop around birth and extend perivascular processes in the postnatal period, could not account for the formation of a functional BBB which occurs much earlier during embryogenesis.[15] Recent works have established the importance of the canonical Wnt/β-catenin pathway and the coordinated role of several Wnt members secreted by neural progenitors in the growth of neovessels invading the neural tube. This Wnt pathway also promotes the expression of selective proteins (e.g., claudin 5, glucose transporter 1, see below) that are part of the CNS-specific endothelial phenotype.[16−18] Daneman and collaborators have confirmed and extended pioneer work by Hellstrom et al.[19] by showing in both rat and mouse that the subsequent establishment of the functional integrity of the BBB was coincident with the recruitment of pericytes around these growing vessels around embryonic day 12.[20] Using mice with hypomorphic alleles of the platelet derived growth factor receptor B gene, resulting in various numbers of pericytes, the authors observed an inverse correlation between pericyte coverage of the vascular endothelium and BBB permeability to biotin, a small polar compound (0.5 kDa), in neonates. Using a comparable approach of partial and viable pericyte deficiency in mouse, Armulik and collaborators extended these findings to the BBB of young adult animals.[21] They similarly observed an increased permeability to a large range of tracers and to water in pericyte-deficient animals. Both studies reported that, while the expression of BBB markers such as tight junction proteins (e.g., claudin 5), influx transporters (e.g., Glut1) or efflux transporters (e.g., P-glycoprotein) is not altered, the lack of pericyte results in ultrastructural abnormalities in tight junctions and increased transcytotic activity of the endothelial cells, which could contribute to the leakiness of the barrier. These findings are consistent with the more general observation that pericyte density and coverage of the abluminal endothelial surface, which vary among organs, correlate positively with endothelial barrier properties. However, density may not be the only determinant by which pericytes regulate BBB permeability and maintenance. Evidence for brain-specific markers of pericytes, involved in ion transport and intercellular signaling, suggest that these cells may signal to the cerebral endothelium in a different manner compared to other vessels (discussed in ref 22).

Although astrocytes cannot account for early embryonic vasculogenesis or induction of the BBB phenotype and integrity at that time, these cells may still have major functions in the postnatal expansion of the vascular network and BBB maturation, or later in the maintenance or restoration of BBB integrity as suggested by previous in vitro studies. In vivo evidence that astrocytes regulate brain angiogenesis has been provided recently using mice conditionally deleted in glial progenitors. The severe reduction in cortical astrocyte number observed in the brain at the early postnatal period resulted in reduced growth and branching of blood vessels.[23] Sonic hedgehog has been proposed as one of the soluble factors involved in the cross talk between astrocytes and endothelial cells within the NVU.[24] It is produced by astrocytes and promotes barrier properties in cultured brain endothelial cells which express the receptor and downstream genes of the signaling cascade . Pharmacological inhibition of the hedgehog pathway in adult mice resulted in an increased permeability to fibrinogen and dextran.

The astrocytic end-foot which represents the site of gliovascular communication is a specialized membrane domain of the cell, enriched in various membrane proteins, such as connexins, channels, and transporters, some of them involved in the control of water permeability, namely, aquaporin 4.[25] In mice deficient in astroglial connexins, astrocytic end-feet displayed morphological, structural and biochemical abnormalities. These alterations were concurrent with an increase in BBB permeability to proteins in conditions of high vascular pressure.[26] This indicates that maintenance of the BBB integrity can be compromised by deficient astrocytes. Communications between cells of the neurovascular unit are obviously more complex than just bipartite interactions. Besides increasing BBB permeability, pericyte deficiency also affected the phenotype of astrocytes in the adult brain,[21] and altered the polarity of the astroglial end-foot, namely, the distribution of aquaporin 4. Given the highly intricate physical and biochemical connection network within the neurovascular unit, it is likely that defect in any of the cell components will affect its neighbors and impact on the barrier integrity.

2.2. The Blood−CSF Barrier. In vertebrates, choroid plexuses are papillary structures organized as multiple villi and projecting into the intracerebral cavities. They occur in each of the four major cisternae, developing from the median wall of each lateral ventricle, and from the roof of the third and fourth ventricles. Although the three types of choroid plexuses display different macroscopic morphologies, they share a common histological organization. They consist, at the CSF-facing surface, of a single cuboidal epithelial layer deriving from the ependyma, and an inner core of loose and highly vascularized connective tissue, originating from the leptomeninges (Figure 1). The choroidal epithelial cells differ fundamentally from the adjacent ependymal cells by the presence of apical beltlike tight junctions and, therefore, form the anatomic basis for the restrictive BCSFB.[27] As a consequence, the vascular bed in the stroma lies out of the CNS, and the unusually nonsinusoidal but large capillaries supplying each villus are to be differentiated from the BBB-forming capillaries. The thin endothelium, resting on a basal membrane, displays diaphragmed fenestra-

tions which, in secretory tissues, presumably represent membrane areas highly permeable to fluid and solutes, including proteins (see section 3). Tracer studies using horseradish peroxidase or microperoxidase have revealed the absence of a barrier between the blood and the extracellular space of the stroma.[28] Distinctive features of the choroidal epithelium, which are typical of secreting/reabsorbing epithelia, include (i) large numbers of pinocytotic-like vesicles (see below), (ii) a high number of mitochondria assumed to provide a high respiratory metabolism capacity and the energy required for maintaining ionic gradients and for fluid secretion,[29] (iii) elaborate infoldings and interdigitations found at the basal portion of the intercellular spaces, and (iv) a tightly packed border of microvilli at the apical surface resulting in 5- to 13-fold amplification in the brush border membrane area as measured in five primate species.[29] The apical surface of the choroidal epithelium is also decorated with multiple primary motile cilia.[30] Their function has been recently clarified using cilia-deficient mice, mutated in the intraflagellar transport protein Polaris/Ift188. These mice develop hydrocephalus, whose primary cause is not the shorter size and disorganized beating of motile cilia in the ependyma leading to disrupted CSF flow, but rather an abnormal regulation of ion transport and water secretion across the choroidal epithelium.[31,32] Investigation of the molecular mechanism showed that the choroidal cilia act as sensitive sensors to regulate water transfer and overall CSF production via an autocrine neuropeptide FF-mediated signaling pathway.[33]

The three types of choroid plexuses emerge early during CNS development, between embryonic days 12 and 14 in rat, or embryonic weeks 6 and 7 in human.[34] Interactions between the embryonic neuroepithelium and the underlying primitive mesenchyme contribute to the phenotype of the choroidal epithelium (reviewed in ref 6). The development of all choroid plexuses is regulated by the embryonic dorsal midline, and specified by secreted signaling molecules, among which bone morphogenic proteins play a crucial role.[35,36] A crucial role of the signaling molecule Sonic hedgehog for the coordinated growth of the epithelial and the vascular elements at the embryonic stage was recently established using conditional mutants.[37,38] It implies both paracrine and autocrine pathways. Sonic hedgehog is secreted by the first differentiated choroidal epithelial cells to act on (1) mesenchymal elements (presumably pericytes), resulting in endothelial cell proliferation, and (2) epithelial cells progenitors, localized at the root of the plexus to induce epithelial proliferation and expansion of the tissue.

The choroidal epithelium is a source of numerous growth factors and signaling molecules, and also bears a number of receptors for these compounds,[39,40] suggesting a manifold of autocrine and paracrine regulatory mechanisms. An example is provided by the spatiotemporal pattern of expression of insulin-like growth factor II and its binding sites.[41,42] Insulin-like growth factor II is produced first in the prospective choroidal mesenchyme; its synthesis then shifts to the epithelial layer during choroid plexus development. This and the high density of receptor binding sites displayed by fetal choroidal plexuses suggest that insulin-like growth factor II first acts in a paracrine, and later in an autocrine, manner, to induce the differentiated phenotype of the BCSFB forming cells, such as the expression of transthyretin.[41] The choroidal epithelial cells are also responsible for establishing and maintaining the diaphragmed fenestrations in the choroidal vessels through a vascular endothelial growth factor-dependent pathway.[43,44] This factor is produced by the epithelial cells and selectively secreted at their basolateral membrane (unpublished results).

3. STRUCTURAL FEATURES OF THE BARRIERS

3.1. The Tight Junctions. Tight junctions are located at the most luminal side of intercellular junctional complexes in epithelia. They constitute a network of anastomosing strands in which outer leaflets of adjacent cell membrane fuse, and physically impede the paracellular pathway, allowing only a strictly regulated passage of selected inorganic ions, charged and noncharged solutes, and water via this route. Besides this restricting function toward molecular flux through the paracellular pathway, the tight junctions also serve an organizing role in the polarization of the barrier cells and maintain the asymmetrical distribution of transport systems among the luminal and abluminal, or apical and basolateral membrane domains of the microvascular endothelial or choroidal epithelial cells, respectively.

Molecular identification of the tight junction constituents has come up with a very complex assembly of integral membrane proteins and a manifold of intracellular partners. The latter include scaffolding proteins, such as the ZO-proteins ZO-1, ZO-2, ZO-3 and cingulin, which both bind to the cytoplasmic domain of the membrane proteins and interact with F-actin, thereby recruiting the cytoskeleton to the tight junctions (see ref 45 for a review on tight junction constituents). The tight junction associated membrane proteins comprise occludin, tricellulin, an occludin-related protein which localizes mostly in epithelial tricellular tight junctions, JAMs (junction-associated molecules of the immunoglobulin superfamily), and claudins. Loss-of-function and gain-of-function studies have shown that these claudins contribute to the tight junction strand structure (see for example refs 46−48). Claudins belong to a large family of more than 20 members, and have provided a key explanation to the large range of permeability and electrical resistance measured among epithelia and endothelia in the body, that did not correlate with differences in tight junction ultrastucture (i.e., number and branching of strands observed by freeze−fracture electron microscopy,[49]). Indeed, the subtype of the claudins, the way they homo- or heteropolymerize, and the type of homo- or heterotypic interaction they establish between adjacent cells will determine the distinctive permeability properties of the tight junction they form (see ref 50 for a review).

The molecular identity of the various claudins in tight junctions of the brain interfaces has been deciphered over the last ten years by gene expression studies, Western blot, or immunohistochemical analysis in different mammal species. These works have revealed that a large number of these proteins are present in the endothelial and epithelial tight junctions. Importantly, they also indicated that the BBB and the BCSFB express distinctive claudin subtype.[51] Claudins specifically present in the BBB include claudin 4, claudin 16, and more importantly claudin 5. This last protein is the major claudin in the BBB in rat, mouse and human.[51−53] It localizes in tight junctions, and appears a critical determinant of their permeability, as shown in claudin 5 knockout mice by the subtle size-selective increase in the brain penetration of tracers.[52] Other claudin genes are expressed in the BBB, and their products were immunodetected in BBB tight junctions, such as claudin 3 or 12, without displaying a distinct BBB specificity.[51,52] The members most highly expressed in the

BCSFB are claudins 1, 2, and 3. They all distribute at epithelial tight junctions in the adult rat choroid plexus, as well as in human tissue.[51,54] Claudins 9, 19, and 22 are also selectively enriched in choroid plexuses and, with the exception of claudin 22, have been detected in the rat choroidal epithelial junctions by immunochemistry.[51]

Claudins have been classified in two categories, the pore-forming and the barrier-tightening claudins, on the basis of their effect on transcellular electrical resistance in transfected cultured cells, or on the basis of human deletion phenotypes (see ref 50 for a review). It is remarkable that the BCSFB and the BBB tight junctions comprise distinctive claudins that respectively belong to the first and the second categories, such as claudin 2 in the choroidal epithelium and claudin 5 in the endothelial barrier. The differential expression of claudin subtypes between the barriers may finally contribute to explain the prevailing concept of "tighter" tight junctions in the BBB and "leakier" tight junctions in the BCSFB, and might be the reason for the differential permeation by lanthanum of rabbit brain capillary and choroid plexus tight junctions.[55] The barrier specificity in claudin proteins can be related to the respective functions of these interfaces in terms of ion regulation. Claudin 2 has been characterized as a monovalent cation selective channel, with a great specificity for K^+, as well as a paracellular water channel.[50,56,57] As such, it may represent in the BCSFB a key element contributing to one major function of this interface, i.e., CSF secretion (discussed in ref 51).

Importantly, the selective junctional proteins, claudin 5 in the endothelial barrier or claudins 1 and 3 at the choroidal epithelium are expressed and localized at the junctions from early embryonic development (Figure 4). Immunochemical evidence for claudin 5 has been presented in mouse brain endothelial cell junctions from embryonic day 12, when cortical angiogenesis begins,[20,58] and in human fetuses of 14 weeks.[53] Similarly, claudins 1 and 3, and claudin 2 with a more heterogeneous pattern, are detected in human choroidal epithelial junctions in fetuses from 8 weeks of development, i.e., very rapidly after the choroid plexus primordium starts to differentiate.[51,59] Permeability studies using small labeled polar tracers (fluorescent dextrans) showed that both BBB and BCSFB junctions are functionally effective at such early times, and capable of impeding the paracellular flux of the compounds.[60,61]

3.2. Pinocytosis at Blood–Brain Interfaces. Pinocytosis results from the invagination of plasma membrane domains ultimately leading to the formation and traffic in the cytoplasm of vesicles whose diameter ranges between 50 and 120 nm. This process can lead to the delivery of selected solutes to the cells or to the degradation of the vesicular content into the lysosomal compartment. It can also result in transcytotic transfer of the cargo carried by the vesicle, thus explaining why pinocytosis can participate in the overall permeability of epithelia and endothelia. Vesicles can form from clathrin-coated pits which are enriched in various cell surface receptors supporting receptor mediated endocytosis. Other vesicles originate from caveolin-1-containing lipid rafts. They are also engaged in receptor-mediated transcytosis, and, in peripheral endothelial cells, in fluid phase endocytosis. A third type of vesicles forms from non-clathrin-, non-caveolin-coated lipid rafts.

The number of vesicles is very limited in endothelial cells forming the BBB, compared to endothelial cells of permeable vessels found in the choroid plexus, area postrema or muscle.[62]

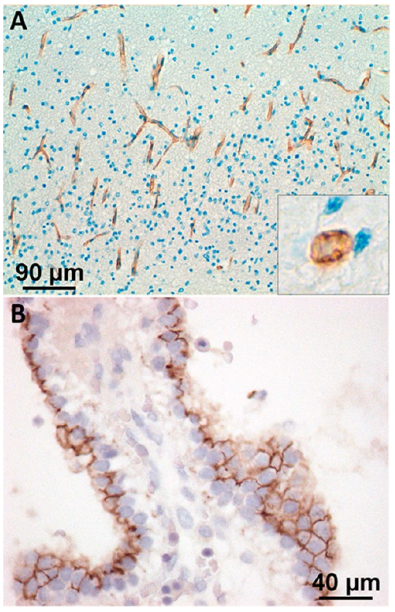

Figure 4. Tight junctions that seal barrier cells together develop early during development. (A) Brain microvessels are strongly immunoreactive for claudin 5 in a human 18-week-old fetus. Inset shows a capillary cross section. (B) Claudin 1 immunolabeling is already intense and continuous around choroid plexus epithelial cells in a human 8-week-old fetus. The lateral ventricle choroid plexus is shown. Rabbit polyclonal anti-Cld-1 Ab (51–9000) and mouse monoclonal anti-Cld-5 antibody (mAb) (35–2500) (Zymed Laboratories, Invitrogen, Carlsbad, CA) were used in A and B, respectively. Methods as described in Kratzer et al.[51]

This has been confirmed in a wide range of species in several studies (reviewed in ref 63). The number of non-clathrin-coated vesicles is especially low and fluid-phase endocytosis is very limited, at least in adult brain endothelium and in nonpathologic conditions. Thus, the receptor mediated transport processes that are being described in brain endothelial cells (see below) can only be accounted for by this low number of vesicles present in the barrier. Transcytosis can also be enhanced through adsorptive endocytosis induced by chemically transformed cationic proteins or peptides, and by large cationic pharmaceutical compounds. Of note, a developmental downregulation of pinocytosis involving both clathrin-coated and caveolin-1-coated vesicles exists at the BBB, during the fetal period, paralleling the appearance of the perivascular pericyte coverage (refs 15,64 and section 2).

In contrast to the BBB forming endothelium, choroidal epithelial cells harbor large numbers of vesicles, including both clathrin-coated and non-clathrin-coated vesicles. They are found linked to the basolateral infolding membranes, throughout the cytoplasm, and at the base of the apical microvilli (ref 65 and Figure 5). Their abundance in the rat choroidal epithelium does not seem to vary between developing and adult animals (unpublished observation). The BCSFB therefore presents a strong endocytotic activity, which is likely related to the vigorous metabolic and synthetic functions of the choroid plexus. The exact relevance of this vesicular abundance to the transcellular transport of proteins and other macromolecules between blood and CSF in either direction remains elusive, at least for fluid-phase endocytosis as investigated with HRP.[66,67] Receptor-mediated transcytosis of selected macromolecules may however take place at the BCSFB, and in some

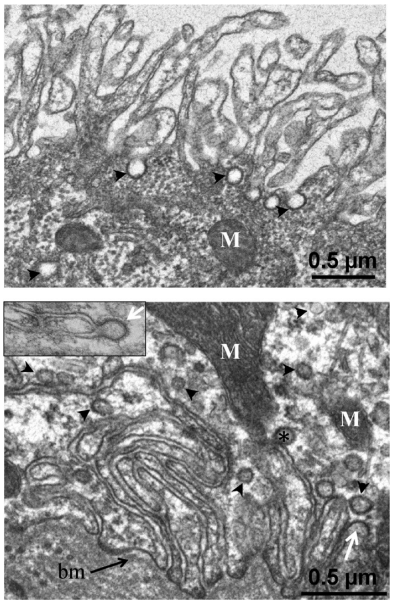

Figure 5. Vesicular traffic at the basolateral and apical membranes of the choroid plexus epithelial cells. Lower panel: Transmission electron micrographs of both adult and developing rat choroid plexuses show numerous vesicles (e.g., arrowheads), many of them coated, in close proximity to the basolateral membrane infoldings of epithelial cells. White arrows in main picture (adult) and inset (newborn) point to coated pits forming prior to vesicle detachment in cells of lateral ventricle choroid plexus. Star indicates a vesicle still bound to the membrane infolding. Upper panel: vesicles are seen at the apical pole of epithelial cells in lateral ventricle choroid plexus from a newborn rat (arrowheads). Many coated vesicles are forming at the base of apical cytoplasmic processes. Numerous vesicles, of both the coated and noncoated types, are seen throughout the cytoplasm, and a number of vesicles also bud from the lateral membranes of the choroidal cells (not shown). Abbreviations: bm, basal membrane; M, mitochondria.

instances differ between developing and adult individuals (see section 5).

4. REGULATION OF MOLECULAR FLUX ACROSS THE BLOOD–BRAIN INTERFACES: TRANSPORTERS, RECEPTORS AND ENZYMES

Despite the specific restrictive morphological features harbored by the blood–brain interfaces, both the cerebral endothelium and choroidal epithelium are dynamic interfaces through which a heavy, yet regulated molecular traffic occurs.

4.1. Inorganic Ions. The integrity of blood–brain interfaces is central to the local maintenance of inorganic ion homeostasis necessary to neuronal activity. Like other cells, cerebral endothelial and choroidal epithelial cells harbor monovalent inorganic ion transporters and channels necessary to regulate the cell volume. In the cerebral endothelium, the Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) and the Na^+-K^+-ATPase located at the luminal and abluminal membranes, respectively, are main actors of this regulation. The transcellular and paracellular BBB permeability to monovalent ions remains however very limited in physiological conditions. Only in pathological conditions such as ischemia, an increase in BBB permeability to ions is observed, which results from changes in expression of different channels and transporters. It leads to the ionic edema which follows cytotoxic edema.[68] An increase in the BBB permeability to inorganic ions is also thought to play a role in seizure generated by epilepsy.[69] At the BCSFB, besides their role in epithelial cell volume regulation, channels and transporters for monovalent inorganic ions control CSF secretion. This secretion results from a coordinated action of different monovalent inorganic anion and cation transporters, exchangers and channels leading to transcellular fluxes of Na^+, Cl^- and HCO_3^- from the blood to the CSF, and promoting water movement across the choroidal epithelial monolayer. The whole process is driven by the apically located Na^+-K^+-ATPase and carbonic anhydrase II. Current understanding of CSF secretion implies that only the transfer of K^+, necessary to provide adequate K^+ levels in the CSF, occurs via the paracellular route.[70]

The blood–brain interfaces also supply the brain with multivalent inorganic elements such as Ca^{2+} and Mn^{2+}, that are necessary to basal neural cellular functions and micronutrients. These two cations are transported in particular by an active and unidirectional process across the blood–CSF barrier, which thus appears to play a specific role in the delivery of divalent metals to the brain. The molecular determinant(s) involved in this transport remain(s) to be precisely deciphered (discussed in ref 71). In addition specific proteins such a transferrin can carry other inorganic anions such as iron or Mn^{3+}. The delivery of such carrier protein to the brain is discussed below.

4.2. Small Organic Compounds. Exchanges of small organic compounds between blood and brain are regulated by plasma membrane transporters working either in the blood-to-brain direction or in the brain-to-blood direction, or both. The directionality of transport is set by the subcellular location of the transport system (blood-facing or brain-facing membrane of the endothelial and epithelial cells), and by the transport mechanism which can be primarily energy-dependent (ATP binding cassette (ABC) transporters), secondarily energy-dependent (solute carrier (SLC) coupled to Na^+ transport, and thus to Na^+-K^+-ATPase) or via facilitated diffusion (SLC transporting their substrates along the concentration gradient).

Various SLC transporters are involved in the supply of nutrients that are too hydrophilic to diffuse across the brain interfaces.[72] At the BBB, Glut-1 (SLC2A1) is responsible for providing glucose to the neural cells. Essential large neutral amino acids are transported mainly by LAT1 (SLC7A5) and cationic amino acids by the Na^+-dependent system y^+ (SLC7A1), respectively. Other amino acid transporters such as LAT2 (SLC7A6), system A (alanine preferring, SLC38A2), system ASC (alanine, serine, cysteine preferring, SLC1A5), system Bo^+ (basic amino acid preferring, SLC7A3) or system N (glutamine preferring, SLC38A5) are active at the BBB, although their directionality of transport and exact function remain incompletely understood. Glucose and selected amino acid transporters are also present at the BCSFB, where they may be more dedicated to the delivery of nutrients to the metabolically active choroidal epithelial cells themselves, rather than to CSF and brain, at least in adult individuals (references in ref 72). The monocarboxylate transporter MCT1 (SLC16A1) located at the BBB removes the excess of lactate produced by brain astrocytes following glucose metabolism. It can also function as an influx transporter in the developing brain, during ketogenic diet periods, or following hypoxic insults, by extracting ketone bodies from the plasma as a source of energy (reviewed in ref 72). Hormones and micronutrients too are transported through the blood–brain interfaces. Ascorbic acid, for instance, is actively and specifically transported into the brain across the BCSFB by the sodium-dependent vitamin C transporter-2 (SLC23A1).[73,74] As another

example, the cerebral availability of thyroid hormones depends on a complex interplay between blood-to-brain transport by carriers that include MCT8 (SLC16A2), oatp14 (SLC21A14), located at both barriers, and binding to transthyretin, a protein synthesized in larger amounts by the choroid plexuses and secreted into the CSF.[75,76]

Other substrate-specific transporters are dedicated to the clearance of intracerebrally produced endogenous metabolites across the barriers. Exitotoxic amino acids such as glutamate are released during neurotransmission and are produced in excess in certain situations such as hypoxia. These metabolites are actively transported into the blood by EAA transporters (SLC1A1-3) located on the abluminal membrane of the brain endothelium.[77,78] PEPT2 (SLC15A2), specifically localized at the apical membrane of the choroidal epithelium, is mainly responsible for the efflux from the brain to the blood of di- and tripeptides. This system also transports peptidomimetic drugs.[79]

The brain is also facing a large range of small organic compounds that are lipophilic enough to cross the cellular membranes of barrier forming cells. These molecules include potentially toxic xenobiotics such as environmental pollutants, and pharmacologically active drugs. Their brain penetration is controlled and limited by yet another set of efflux transporters that belong to the ABC protein superfamily, or to distinctive SLC subfamilies. These efflux transport systems each display a different but broad and partially overlapping substrate specificity, and can work in concert at both membranes to generate a high level of neuroprotection.[80,81] Relevant ABC transporters include ABCB1 (P-glycoprotein), ABCG2 (breast cancer resistance protein), and several members of the ABCC (multidrug-related resistance proteins) family. Located at the blood-facing membranes, they prevent the brain penetration of their substrates by an energy-dependent, unidirectional, outwardly directed transport mechanism. They accept many structurally unrelated lipophilic and amphiphilic compounds including immunosuppressor, antiretroviral, and antitumor drugs, some antibiotic, antiepileptic, antidepressant and psychotropic agents, and drug conjugates in the case of ABCCs.[80,82−86] Organic anion and cation transporters of the SLC21 family such as oatp3 (Slc21a7 in rodent), and of the SLC22 family, in particular OAT3 (SLC22a8), display a broad specificity and also participate to the efflux of xenobiotics from the brain. They accept many drugs such as nonsteroidal anti-inflammatory, antibiotic, nucleoside-based antiviral and some antiepileptic agents as substrates. Drug transport mediated by SLC22a8 and Slc21a7 is usually inwardly directed, thus mediating cellular uptake. As these transporters are located at the brain-facing membranes of the barrier cells, they operate by removing drugs from the brain fluid compartments.[87,88] These multispecific efflux transporters are differently distributed between the BBB and BCSFB. For instance, in laboratory animals as in human, ABCB1 and ABCC1 are landmarks of brain microvessels and choroid plexuses, respectively.[89] ABCG2 expression is very high in human brain microvessels,[90] while Slc21a7 is only found at the choroid plexus. Thus the BBB and the BCSFB achieve their neuroprotective functions by barrier-specific mechanisms, possibly related to the nature of the environment protected by the blood−brain interfaces, which is more lipophilic for the BBB than for the BCSFB (discussed in ref 89).

Another barrier-related facet of the blood−brain interfaces is provided by metabolizing enzymes. This was discovered decades ago for endogenous amine neurotransmitters.[91−93] Enzymes that transform drugs and xenobiotics into hydrophilic and usually less active metabolites have also been identified in both the rodent and human BBB and BCSFB.[78,90,94,95] These enzymes include cytochrome P-450 dependent monooxygenases that add hydroxyl functional groups to their substrates, epoxide hydrolases that inactivate toxic electrophilic epoxides, and several conjugating enzymes that couple their substrates to glucuronic acid, sulfate or glutathione moieties. The activity of several drug metabolizing enzymes is especially high in the choroid plexus. Evidence that these enzyme activities influence the cerebral bioavailability of drugs has been gathered so far only for some choroidal enzymes. The use of a differentiated cellular model of the BCSFB and of isolated choroid plexus in survival medium allowed to show that glucuronic acid or glutathione conjugation in rat choroidal epithelial cells, coupled to a basolateral (blood-facing) efflux of the formed conjugates, likely mediated by ABCC proteins, confer to the BCSFB an efficient function of metabolic barrier that limits substrate entry in CSF.[96,97] The high glutathione *S*-transferase and sulfotransferase activities and ABCC levels measured in human choroid plexuses[89,96,98] suggest that similar mechanisms occur in human.

These transport and metabolic barrier mechanisms can be deregulated in different CNS diseases such as epilepsy, ischemia, and inflammation. Evidence that they can be pharmacologically enhanced has also been brought forward (reviewed in ref 5). Polypeptides and macromolecules are usually not substrates for efflux transporters or detoxifying enzymes, with the possible exception of selected ones such as the ∼4 kDa amyloid β-peptide which to some extent may be transported out of the brain by ABCB1.[99]

4.3. Polypeptides and Proteins. The fate of biologically active small peptides in the context of brain delivery remains elusive. While low blood-to-brain influxes have been described for compounds such as enkephalin or arginin vasopressin, efflux mechanisms appear to dominate over influx at the BBB for most of them. This indicates that the net brain entry is limited for these compounds, and the physiological significance of this low entry remains to be ascertained (reviewed in ref 72). It also suggests that efflux transport blocking strategies can be envisioned to improve the delivery of neuroprotective small peptides. For instance blocking PTS-6 efflux pump by an antisense strategy increases the cerebral delivery of a 27 amino acid truncated form of pituitary adenylate cyclase-activating polypeptide (PACAP) which has potent neuroprotective properties. It increases the drug efficacy in reducing the infarct size in a mouse stroke model, and in improving cognition in a model of Alzheimer's disease.[100]

Larger polypeptides, proteins and lipoproteins also enter the brain endothelial cells by receptor-mediated endocytosis, and to some extent can reach the brain side using a transcytosis mechanism. Receptor mediated endocytosis and possibly transcytosis pathways have been largely described and discussed in particular for insulin receptor, transferrin receptor, low density lipoprotein receptor (reviewed in refs 63,101), and more recently low density lipoprotein receptor-related protein 1 (LRP1). While binding of amyloid beta peptide to this receptor at the abluminal membrane of the endothelial cell is an accepted mechanism contributing to the elimination of this peptide from the brain,[102] LRP1 involvement in the influx of insulin-like growth factor I into the brain has been shown in vitro.[103] These receptor-mediated endo/transcytotic pathways

are currently used in strategies aiming at improving macromolecules delivery to the brain (e.g., ref 104). Conversely efflux mechanisms have been described for macromolecules such as immunoglobulins G (IgGs). A Fc receptor-dependent mechanism, located at the BBB, was shown to efficiently transport intracerebrally infused IgGs into the blood, thus strongly reducing their cerebral half-life.[105] The exact significance of this mechanism as a factor setting the cerebral bioavailability of circulating therapeutic IgGs remains a matter of debate (see ref 106 and section 5.3).

Although explored to a lesser extent, receptor-mediated endocytosis and transcytosis also occur at the BCSFB, together with other protein transfer mechanisms. Understanding these transport pathways can lead to the development of new delivery pathways to the CSF and brain for macromolecules, especially in the context of pediatric treatment. The next section will focus on these mechanisms in the BCSFB.

5. MACROMOLECULAR TRANSPORT IN THE CENTRAL NERVOUS SYSTEM ACROSS THE BLOOD–CEREBROSPINAL FLUID BARRIER

It is important to remember that the choroidal endothelium has no restrictive effect on large molecule transfer between blood and CSF. This has been clearly established by ultrastructual studies, showing that proteins can quickly equilibrate in the stromal compartment and fill the basolateral intercellular spaces up to the tight junction complexes. Horseradish peroxidase injected intravenously in rat readily passes the capillary wall (e.g., ref 107). Immunohistochemical analyses of plasma proteins consistently show a marked staining of the stromal space in addition to the vascular lumen (e.g., 108). This lack of restriction is a common feature of the nonsinusoidal fenestrated microvasculature such as those of the pancreas, adrenal cortex and kidney peritubular capillaries (reviewed in ref 109). In electron microscopy ultrastructure studies, diaphragmed fenestrations appear as areas of extreme attenuation of the cell cytoplasm, with an average length of 70 nm. In freeze–fracture replicas, these fenestrations appear as flowerlike structures and are composed of a central membranous diaphragm from which several fibrous septae radiate toward the external rim.[110] These fibrils create wedge-shaped channels, whose arc width determines the upper limit of the pore size. In choroid plexus capillaries, this upper limit has been estimated close to 12 nm, i.e., approximately the diameter of ferritin, a large protein of 800 kDa.[109] Blood-to-CSF transport of macromolecules across the choroid plexus is thus primarily dependent on existing transcellular mechanisms in the choroidal epithelial cells.

This section will summarize our current knowledge on these mechanisms, focusing first on the transport of plasma proteins via a selected subpopulation of choroidal epithelial cells. It will then explore various transcytotic processes, involving canonical receptors, that may be functional at the BCSFB, and finally examine the issue of immunoglobulin transfer.

5.1. Transport of Plasma Proteins across the Blood–CSF Barrier. Preliminary evidence of plasma protein transfer from blood to CSF using labeled albumin and immunoglobulins in newborn rats was presented in 1976, and was at that time interpreted as a result of barrier immaturity.[111] Since then, the existence of a developmentally regulated specific mechanism of protein transfer across the choroidal epithelium has been largely sustained. First, immunochemical studies clearly demonstrated the presence of plasma protein positive cells in the epithelium of fetal choroid plexuses in human and various mammal species.[108,112,113] Second, the apparent specificity of the transcellular route for individual proteins and the lack of correlation between CSF/plasma concentration ratios and the molecular radius of these proteins suggested that receptor-mediated transfer is involved in this pathway, especially during early stages of brain development.[112,114] Third, protein transfer from blood to CSF was confirmed using exogenous plasma protein, such as human albumin in immature sheep fetuses or immature rat pups.[114,115] It has been proposed that this specific intracellular route accounts for the high protein concentration in CSF during fetal life (e.g., ref 113).

The detailed investigation of plasma protein immunoreactive cells revealed that only a small proportion of the epithelium is stained (e.g., Figure 6 for albumin). Immunopositive cells

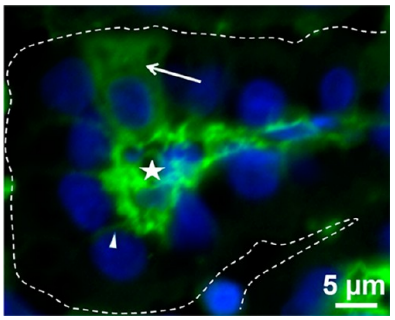

Figure 6. Selected cells of the blood–CSF barrier are immunopositive for plasma proteins. Albumin immunoreactivity in fourth ventricle choroid plexus of 2-day-old rat. The dashed line delineates a choroidal villus. The arrow points to an immunopositive choroidal epithelial cell. The stroma of the villus (star) is filled with albumin originating from the fenestrated capillaries and diffusing in the intercellular space between adjacent choroidal epithelial cells up to the apical junction (arrowhead). Paraffin embedded sections, blocked with gelatin, were incubated with a sheep polyclonal anti-albumin antibody (Abcam ab8940), followed by secondary Alexa 488-conjugated anti-sheep antibodies. Nuclei were stained with DAPI.

appear very rapidly after the choroid plexus starts to differentiate. Their number increases during development,[108] although the percentage of transporting cells decreases because of substantial choroid plexus growth. In *Monodelphis*, protein transporting cells represent about 10% of the total epithelial cell population up to 9 days after birth (equivalent to newborn rat). Their abundance then slowly decreases to reach the adult value of about 4–5% by day 45. Therefore, the absolute protein transfer capacity of the BCSFB may actually be larger in the adult brain, in contrast with the concurrent drop in CSF protein concentration. This apparent discrepancy has been explained by several factors, including a substantial increase in CSF volume and turnover rate.[116] Not all plasma proteins can be detected within choroidal epithelial cells, indicating that the mechanism of cellular uptake is selective for certain proteins.[117] In early fetal stages, the transporting cells display a rather high degree of protein specificity that they tend to lose during development.[108]

Transfer of plasma proteins across the choroid plexus seems to be unidirectional, functioning only from blood to CSF. This was revealed by the accumulation of bovine fetuin in a subpopulation of choroidal epithelial cells following intraperitonal injection of the exogenous protein in *Monodelphis* fetuses, and the lack of cellular uptake after intraventricular

administration of the same molecule.[108] This is in contrast to older studies showing stromal penetration of fluorescent albumin, either in vivo after intraventricular perfusion in adult cat or ex vivo using isolated choroid plexuses of immature chick.[118] This discrepancy might be related to several factors. These include the species (*Monodelphis* vs cat and chick), the developmental stage (*Monodelphis* fetus vs adult cat), the proteins (fetuin vs albumin) which could use specific recognition mechanisms, or the experimental setting (*Monodelphis* in vivo vs chick ex vivo). A high uptake of polar tracers of comparable size (fluorescent dextrans) also occurs in selected choroidal epithelial cells. This uptake is however seen from both the basolateral and the apical cell surface.[108] In addition, staining for either the plasma proteins or the polar tracers does not necessarily colocalize in developing choroid plexus. These two categories of compounds seem therefore to use different transcellular pathways, at least in early fetal stages.

A recent transcriptomic analysis of the mouse choroid plexus, comparing embryonic and adult stages, has provided potential plasma protein-binding candidates.[119] Among those are the membrane proteins glycophorin A and C, and the soluble protein SPARC/osteonectin, all known to have an albumin-binding capacity. All three genes are expressed at higher levels in embryonic than in adult tissue. Single cell PCR for these putative genes in individual choroidal epithelial cells immunoreactive for albumin or total plasma protein showed a substantial degree of cellular colocalization. On the basis of these results and of the differential subcellular distribution of glycophorin A and Sparc in mouse choroid plexus, the authors proposed that SPARC and glycophorins are involved in the transfer from blood to CSF of albumin and other plasma proteins in the developing choroid plexuses, by playing complementary roles in cellular uptake, intracellular traffic and efflux. They further postulate about the existence of a second nonspecific pathway functioning in the adult, and involving several vesicle-associated membrane proteins (VAMPs) whose expression in choroid plexus is upregulated in adult compared with embryonic mice.

The physiological significance of plasma protein penetration through the choroid plexus into CSF during brain development may be 3-fold. The resulting high protein concentration in CSF could build up an intraventricular osmotic pressure which in turn will drive water transfer, thereby promoting the ventricular expansion required for brain morphogenesis.[120] It is also possible that the plasma proteins themselves are involved in some aspects of brain development, as suggested by their uptake in cells of the ventricular zone.[112] Finally, certain plasma proteins, especially albumin or fetuin, are carriers for biologically active compounds such as hormones, growth factors or morphogens and may contribute to their delivery into the developing CNS.[121] The precise cellular mechanisms supporting plasma protein transport in selected cells of the choroidal epithelium need to be further elucidated. Manipulating this pathway may prove a strategy of interest for macromolecule delivery to the developing and possibly the adult brain.

5.2. Canonical Receptors in Macromolecule Transcytosis across the Blood–CSF Barrier. *5.2.1. The Transferrin Receptor.* The transferrin receptor TfR1 is a homodimer present at the cell membrane surface in a wide range of tissues. It meets the basic iron requirement for cell mitochondrial respiration and cell proliferation. Following binding of iron-loaded transferrin (holoform), the complex is internalized by endocytosis. Vesicle acidification results in iron release from transferrin and export of the metal to the cytosol by the divalent metal transporter DMT1. TfR is then recycled to the cell membrane where iron-free transferrin (apo-form) is released due to its lower affinity for the receptor at neutral pH. Cellular iron can be stored via binding to a high capacity carrier, ferritin.

Brain iron homeostasis is essential as both iron deficiency and iron excess are associated with neuronal dysfunction. Iron delivery to the brain through the transferrin/TfR pathway came from immunochemical evidence of TfR in both blood–brain interfaces in rat.[122,123] TfR is an early phenotypic hallmark of the BBB and is absent from the non-BBB cerebral vessels such as those of circumventricular organs.[124] It has also been identified in human brain microvessels and choroid plexus epithelium.[125] The concept of TfR mediated delivery of iron to the brain implies either the release of cytosolic iron at the brain-facing membrane of the barrier cells or, alternatively, a transcytotic mechanism different from the endocytotic pathway that provides cells with iron for their own need. It is still unclear why, in the latter scenario, the high affinity holo-transferrin would dissociate from the receptor or how iron would leave transferrin for which it has a very high affinity, once the complex reaches the brain-facing membrane. As for the former pathway, free iron could be either released by the membrane transporter ferroportin or secreted in the brain space following binding to intracellularly synthesized transferrin. Transferrin expression in the brain is especially high in the choroid plexus, which is considered as the main source of CSF transferrin.[126,127] Evidence for transferrin gene expression at the BBB has also been obtained by quantitative PCR analysis of brain microvessels isolated from human cortex,[125] and by microarray analysis of RNA from rat cortical microvessels (unpublished results). Importantly, all the other proteins involved in the "classical" endocytotic pathway are expressed in brain microvessel and in choroid plexus.[125]

Whether endocytotic and/or transcytotic mechanisms are involved in iron transfer across the blood–brain interfaces has been the subject of debate. The consensus is that TfR-mediated endocytosis followed by iron release from the cytosol primes over transcytosis at the BBB. The same may be true for the BSCFB. Following intravenous injection of [^{59}Fe-^{125}I]-transferrin, labeled transferrin was recovered in the CSF, but in quantities too low to account for ^{59}Fe penetration.[128] The unidirectional uptake rate of [^{59}Fe]-transferrin, measured in an arterial perfusion model in rat, was more than 100 times higher in the choroidal tissue than in CSF, possibly reflecting a significant uptake of the complex in the choroidal epithelium, and a slow release of free or bound iron from the barrier cells into CSF.[129] Altogether, these data suggest that TfR mediates iron delivery across the BCSFB via both transcytotic and endocytotic mechanisms, and that the barrier can play a buffering role for CSF against elevated or low iron plasma levels. Besides, this barrier may also respond to higher brain requirements. Indeed, iron transfer through the choroid plexus may represent a compensatory pathway to face a lower transport capacity of the BBB, as observed in the restless leg syndrome, characterized by brain iron deficiency. It was recently shown in this pathology that the iron management protein machinery, including TfR, transferrin, DMT1, ferroportin, and ferritin, is induced in human choroid plexus, presumably in response to lower intracellular levels of iron.[125]

In this scenario, iron continuously released into the CSF would have to reach its cellular targets in both periventricular and deeper brain structures. Although this has not been

demonstrated specifically for iron, the relevance of the CP-CSF system in the blood-to-brain delivery of divalent metals has been evidenced in vivo for manganese by magnetic resonance imaging (MRI). Owing to its paramagnetic properties, manganese is a contrasting agent and a cerebral activity marker in experimental MRI. It accumulates in the brain with a preferential localization in hippocampus and basal nuclei. Time-course MRI analyses performed in both rodent and non-human primates have shown that, following systemic administration, this metal accumulates into the CSF first, before spreading into the periventricular regions and entering nerve cells, ultimately reaching its final deeper localization via retrograde transport through nerve fibers.[130,131]

5.2.2. The Insulin Receptor. Insulin receptors are widely distributed in peripheral tissues and in the brain, where insulin exerts neurotrophic and local neuromodulatory functions.[132] Insulin has also been identified as one of the adiposity signals involved in the long term regulation of food intake and energy homeostasis (reviewed in ref 133). Peripheral insulin secreted by pancreatic β cells enters the brain to interact with specific receptors primarily on hypothalamic neurons of the arcuate nucleus. Early studies investigating the distribution of insulin receptor binding sites in adult rat or mouse brain all reported a high binding on the choroid plexuses.[134−137] The binding was specific for the insulin receptor as assessed by displacement and competition studies. Computerized densitometric analyses indicated that, among all brain structures, choroid plexus actually displayed the highest density of insulin receptor.[135,137] These data were further confirmed by in situ hybridization which demonstrated high levels of insulin receptor mRNA in choroid plexus.[138] Studies in dog and human indicated that elevated levels of plasma insulin following continuous blood infusion led to increased CSF insulin levels.[139,140] Receptor-mediated transport of insulin across the BBB has been demonstrated, presumably involving the insulin receptor.[141] Whether a similar mechanism is also efficient at the BCSFB remains a matter of debate. Attempts to model the kinetics of insulin uptake in CSF from plasma in dog did not yield a clear and definite answer as to the route by which the hormone gets access to the fluid.[142,143] The data suggested the presence of an intermediate compartment between blood and CSF, but did not allow discriminating whether the compartment represents the parenchymal interstitial fluid or the choroid plexus tissue, or both.

Concurrently, intracerebroventricular perfusion studies in rat have indicated that insulin is cleared from CSF more rapidly than inulin, a marker of CSF bulk flow.[144] This could be related to a specific neural cell uptake of insulin from the CSF, across the ependyma, but it also raised the possibility that choroidal insulin receptors participate in the clearance of the hormone and the termination of its signal. The subcellular localization of the insulin receptor in choroid plexus epithelial cells and the directionality of a potential transcytotic process require more investigations in order to delineate the role played by this abundant choroidal receptor in the cerebral availability of insulin from its function in signaling. The expression or immunolocalization in choroid plexus, of insulin receptor substrates 1 and 2, which are direct downstream effectors of insulin receptor activation,[145,146] support the activity of an insulin signal transduction pathway in this tissue. Such a pathway may be relevant to the control of CSF secretion, a major function of the choroid plexus, as indicated by the insulin receptor mediated regulation of choroidal $5HT_{2C}$ receptor activity.[147] Finally, it will also be important to determine whether and how insulin receptor-mediated endocytosis impacts the brain delivery of antibody based therapeutic agents that target this receptor for transcytosis across the BBB.

5.2.3. The Low Density Lipoprotein Receptor-Related Protein Family. Low-density lipoprotein (LDL) receptor-related proteins (LRPs) constitute a family of cell surface receptors structurally related to the LDL receptor.[148] They are engaged in receptor-mediated endocytosis of a large variety of ligands, and participate in a wide range of physiological processes besides lipoprotein metabolism. Ligands include protease/protease inhibitor complexes and many loaded carrier proteins for hormones or vitamins, such as thyroglobulin, sex-hormone binding protein, transthyretin, retinol binding protein, or else vitamin D binding protein (see refs 148,149 for ligand lists). LRPs also have essential functions in cell signaling through interactions of their cytoplasmic tail with adaptor molecules.[150] LRPs are considered as endocytotic receptors that internalize their ligands and direct them to lysosomes for degradation. For example, LRP2, localized in clathrin-coated pits at the apical membrane of renal proximal tubule cells, mediates the reuptake of filtrated proteins from urine, resulting in lysosomal catabolism and secretion of amino acids in the bloodstream.[151] However, based on recent studies, it has been proposed that LRPs can also mediate transcellular delivery of their cargo across certain barrier-forming cells (e.g., ref 152). This would occur in particular at the BBB, where LRP1 could contribute to the clearance of Alzheimer's amyloid β-peptide from the brain.[102]

Three members of the LRP family have been identified at the BCSFB so far, LRP1 (also known as LRP or α2-macroglobulin receptor), LRP2 (named also megalin), and LRP8 (or apolipoprotein E receptor 2). Although some controversial results exist in the literature, LRP1 appears to distribute in both apical and basolateral membranes and in the cytoplasm of epithelial cells of rat and chicken choroid plexus.[153,154] Importantly, LRP1 immunosignal in rat was the most intense on the choroid plexus epithelium, compared with a large array of peripheral organs or to the other cerebral structures analyzed. This differs from an immunochemical localization study of LRP1 in the human brain which failed to detect the receptor in choroid plexus.[155] This apparent species specificity requires further confirmation given that the same study also failed to reveal LRP1 in the human BBB, in disagreement with more recent works (e.g., ref 156). Direct evidence for LRP1 mediated endocytosis or transcytosis at the BCSFB is lacking. In other tissues, LRP1 has been implicated in various biological processes including lipid metabolism, cell growth, tissue invasion, migration, via binding and internalization of lipoproteins, protease/protease inhibitors complexes, growth factors, extracellular matrix proteins.[148] Many of these polypeptides and proteins are present in the CSF, some of them likely secreted by the choroidal epithelium following local synthesis, such as the protease inhibitor α2-macroglobulin, apolipoprotein E, or the matrix metalloproteases MMP2 and 9.[157−159] Presumably, LRP1 in choroidal cells could accelerate the clearance of protease/α2-macroglobulin complexes from the CSF, and participate in the balance of the proteasic activity of the fluid. Whether it contributes to amyloid β-peptide removal from CSF, as it does at the BBB, is another potential function that requires future investigations.[158]

LRP2 immunoreactivity was demonstrated at the apical membrane of choroid plexus epithelial cells in 3-day-old rat

pup.[160] The apical localization of LRP2 in choroid plexus of the rat pup is in accord with the identification of apical sorting elements in the cytosolic tail of the protein,[161,162] and its apical distribution in many other epithelia.[154] Two independent studies performed on adult rat tissue yielded controversial results.[154,163] The apparent lack of LRP2 reported by Zheng and colleagues may be explained by a lower sensitivity of their technique, and the developmentally regulated expression of this gene in choroid plexus. A quantitative analysis of LRP2 gene expression in rat during development indicated a high level around embryonic day 19 and postnatal day 2, which then substantially decreased in the adult stage (18-fold and 8-fold reduction in choroid plexuses of the lateral and fourth ventricles, respectively, Supporting Information). Noteworthy, LRP2 is expressed on the apical surface of the neuroepithelium during midgestation, where it plays a crucial role in balancing signals provided by Sonic hedgehog and bone morphogenic proteins.[164] Later, LRP2 expressed only in a restricted domain of the ependyma that faces the stem cell niche in the lateral ventricle is engaged in bone morphogenic protein catabolism, and provides the subependymal zone with a microenvironment enabling adult neurogenesis.[165] Relevant to the developmental profile of LRP2 in the choroid plexus, and its high expression during late embryogenesis, one can speculate that the choroidal apical receptor is also engaged in modulating the same signaling pathways, known to contribute to the formation and growth of choroid plexus. With regard to transport activity, recent works have reported that LRP2 may be involved in several transcytotic processes previously described at the BSCFB. First, it had been proposed that the barrier constitutes the route of cerebral entry of leptin, a circulating hormone involved in the hypothalamic control of feeding and energy homeostasis. Leptin transport across the BCSFB has been evaluated in sheep using the ex vivo technique of perfused choroid plexus.[166] Choroidal uptake of iodinated leptin from the perfusion fluid and detection of the hormone in newly formed CSF indicated transcytosis of intact leptin from blood to CSF. Very high levels of RNA coding for a small form of the leptin receptor have been reported in rat and human choroid plexus.[167,168] Because of its short cytoplasmic tail, it was postulated that this receptor function is transcytosis rather than signaling. However, the lack of evidence for leptin transcytosis via this short isoform, and the demonstration that the choroidal receptor is efficient in signaling,[169] did not favor the hypothesis. As leptin is also a ligand for LRP2,[151] the role of the latter receptor in hormone transport was evaluated in vivo in adult rats presenting a partial LRP2 deficiency at the BCSFB induced via intraventricular injection of a lentiviral silencing vector. Results showed a moderate decrease in CSF level of either rat endogenous leptin or human exogenous leptin after intracarotid artery administration, sustaining the involvement, at least partial, of LRP2 in leptin entry into CSF across the choroid plexus.[170] Second, penetration of peripheral insulin-like growth factor I in the CSF was thought to follow a direct route through the BCSFB. This was based on the accumulation of the growth factor in the choroidal tissue following intracarotid administration, and the concurrent and rapid increase of its concentration in CSF.[163] LRP2 contribution in this process was investigated using the lentiviral silencing vector approach. The reduction of LRP2 protein in rat choroid plexus resulted in a statistically significant decrease of the level of labeled insulin-like growth factor I in the CSF.[171] The same study, investigating the effect of this growth factor on LRP2 mediated clearance of amyloid β-peptide, reported that, six months after infusion of the silencing vector, cortical peptide level was higher by comparison with animals treated with an empty vector. Altogether, these different studies infer that, in the BCSFB, LRP2 is able to mediate bidirectional transcytosis, but they also raise a number of issues to be solved. The mechanisms sustaining the switch from the canonical endocytotic/catabolic function of LRP2 to that of a transcytotic receptor are elusive. LRP2 mediates leptin endocytosis and degradation in kidney, but is engaged in leptin transcytosis in choroid plexus. Is this switch cell-dependent, ligand-dependent, or both? How can the apical distribution of LRP2, if proven to be true, be reconciled with its functional activity in blood-to-CSF transcytosis?

Finally, LRP8 expression in choroidal epithelium was first established in rat by in situ hybridization, then assessed in chick by immunohistochemistry, which revealed an exclusively apical localization of the protein.[153,172] The functions of LRP8 at the BCSFB can only be speculated from what is known about its ligands and its functions in other tissues, and from its subcellular distribution. LRP8 shares many ligands with LRP1. It binds and internalizes activated α2-macroglobulin with the same affinity as LRP1, and also binds ApoE.[172] LRP8 could thus have redundant functions and participate in the clearance of α2-macroglobulin complexes from CSF. Another ligand of LRP8 is selenoprotein P, which carries most of the selenium in plasma, and is crucial for homeostasis, storage and delivery of this metal to the whole organism (reviewed in ref 173). In LRP8 knockout mice, brain selenium concentration decreases by more than 50% compared to wild-type mice, although concentrations in the whole body or other organs do not change. How selenium enters the brain is not known, and uptake at the BCSFB seems unlikely given the apical polarization of the receptor. Yet, selenium entry and retention in the brain are clearly determined by LRP8, and the prominent expression at the BCSFB deserves further investigation of this aspect.

5.3. Immunoglobulin Transfer across the Blood–CSF Barrier: Role of the Fc Neonatal Receptor. The major histocompatibility complex class I-related neonatal receptor FcRn is a heterodimer composed of a heavy α-chain associated with β2-microglobulin. Binding of IgGs via their Fc domain to the extracellular region of the receptor α-chain is pH-dependent, and requires a slightly acidic environment. FcRn was identified as the receptor mediating maternal IgG uptake in neonate intestine (see ref 174 for review on FcRn). It remains expressed in the adult in a large number of tissues, especially at mucosal epithelial interfaces. It is responsible for the long serum half-life of IgGs and albumin compared with other serum proteins. Evidence points to the peripheral endothelium or myeloid cells as the main sites responsible for protecting circulating IgGs from catabolism, presumably via an endocytotic/recycling mechanism. The pH dependency of IgG-FcRn interaction implies that initial uptake of IgG from blood is not receptor mediated, but occurs via nonspecific fluid-phase endocytosis. IgG binding to FcRn takes place in acidified endosomes, allowing IgG to be sorted away from lysosomes and recycled to blood.

In other cells, such as epithelial cells lining mucosal interfaces, FcRn mediates IgG transcytosis, thereby participating in immune surveillance and host defense. Extensive evidence for a FcRn-dependent IgG transcytosis pathway has been gathered from in vitro studies in various polarized cell lines (e.g., refs 175–177). This pathway was also documented in vivo in mice, and was shown to be efficient in the female

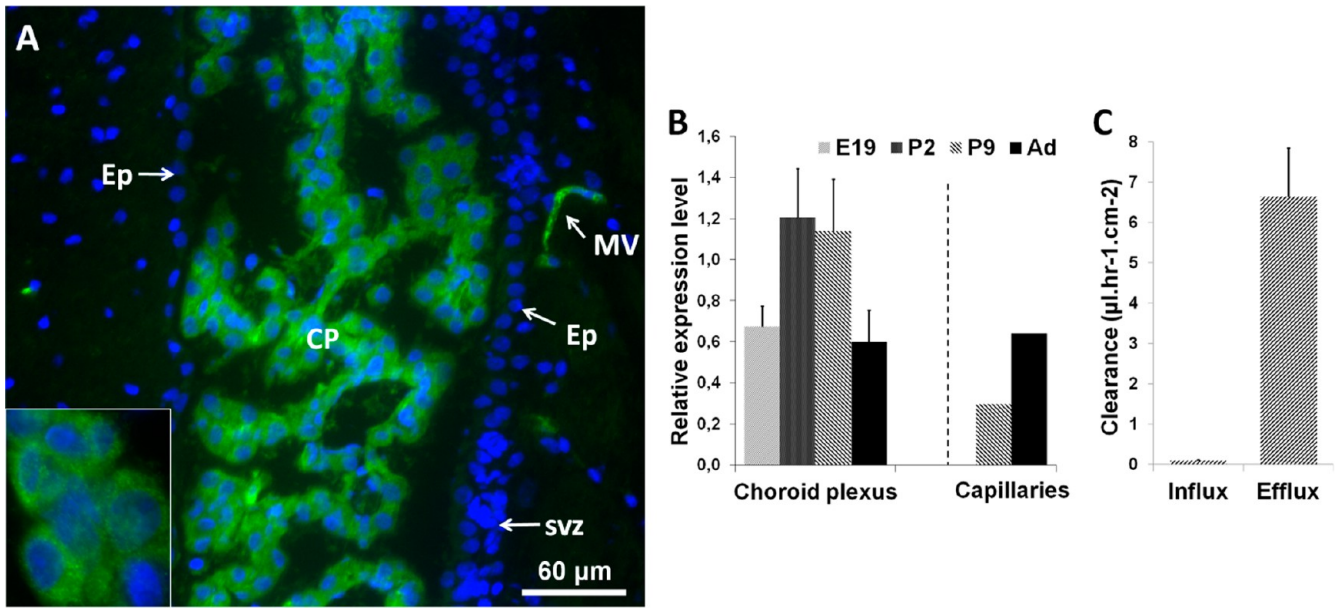

Figure 7. FcRn expression in rat choroid plexus and polarized immunoglobulin transport across the blood−CSF barrier. (A) FcRn immunostaining in 2-day-old rat brain. Both cerebral microvessel and choroidal epithelial cells are intensely labeled. Frozen sections were fixed in 4% formaldehyde and incubated with the mouse monoclonal ant-FcRn antibody IG3, followed by Alexa 488-conjugated anti-mouse antibodies as described by Schlachetzki et al.[180] Abbreviations: CP, choroid plexus; Ep, ependyma; MV, microvessel; svz, subventricular zone. Inset highlights the punctiform immunreactivity suggestive of a vesicular distribution of FcRn. (B) Developmental analysis of rat FcRn transcript levels in choroid plexuses of the lateral ventricle and cerebral microvessels. RT-PCR was performed on tissues sampled at four developmental stages, on embryonic day 19 (E19), on postnatal days 2 and 9 (P2 and P9), and at the adult stage (Ad). Forward and reverse primer sequences are respectively cggagctcaagtttcgattc and gaagcaggccacaaaagaag. Results are expressed relative to a bacterial AraB gene added as an external standard and represent mean ± SD, $n = 4$ for plexuses and mean values of 2 preparations (each from a batch of four animals) for microvessels. Methods as in ref 51. (C) Unidirectional transport of IgGs across choroidal epithelial cells. Transport of 1 μg/mL human IgG (intravenous IgG Clairgyl) was studied in the basolateral to apical (influx) and apical to basolateral (efflux) directions across rat choroidal epithelial cell monolayers grown on Transwell filters as described by Strazielle and Ghersi-Egea.[97] IgG concentrations were measured in both the input and receiving compartments by ELISA (Bethyl Laboratories, Inc., USA). Clearance values were calculated as described by Strazielle and Ghersi-Egea.[97]

genital epithelium as well as the respiratory epithelium of the lung barrier.[178,179]

Immunolocalization studies indicated that FcRn is present in microvascular endothelial cells and in choroidal epithelial cells in both adult[180] and developing (Figure 7A) rat brain. Quantitative gene expression analysis showed comparable mRNA levels in choroid plexuses and microvessels in tissues from adult animals, but revealed a different developmental profile between the interfaces, with a predominant choroidal expression in earlier stages (Figure 7B).

To date, transfer of IgG from blood to CSF across the BCSFB has not been investigated directly. Several lines of evidence point to a developmentally regulated low influx of IgGs, and to the noninvolvement of FcRn in that process. Neonate calves fed with colostrum have a rapid increase (within an hour after initial feeding) in serum and CSF levels of selected proteins including IgGs.[181] Staining of human newborn and fetus choroid plexuses for endogenous IgGs revealed a pattern similar to that seen for other plasma proteins (see section 5.1), with a labeling restricted to selected individual cells.[182] This pattern, contrasting with the homogeneous distribution of FcRn in the choroidal epithelium (Figure 7A), suggests that penetration of IgGs is independent from the receptor, and rather occurs via the plasma protein transport system active in the BCSFB in early development. In support of this, the steady-state CSF to plasma ratio for IgG measured in 3-day-old rat was higher than predicted if transfer was only by diffusion.[115] That difference was no longer seen in 20-day-old animals, consistent with the fact that the pathway is specific to early development. Furthermore, steady-state CSF to plasma ratios were similar for iodinated Fc and Fab fragments in adult mouse, and superior to that of whole IgGs, indicating the noninvolvement of a Fc receptor, and supporting the diffusional process mechanism.[183]

Pharmacokinetic analyses of IgG elimination from CSF following injection in the cisterna magna of monkeys showed a biphasic clearance, with an initial rapid turnover, and a late-phase slow component.[184,185] The latter phase likely results from CSF bulk flow combined with retention at the *glia limitans* and perivascular membranes, and slow release from these sites into the subarachnoid CSF. Given the proximity of the fourth ventricle choroid plexus to the site of injection, one can speculate that the initial rapid clearance phase involves a direct elimination from the CSF across the choroid plexus in addition to diffusion into brain tissue and efflux across the BBB. Transport studies performed in vitro across polarized choroidal epithelial cells in both directions indicated that IgG transcytosis is a unidirectional process (Figure 7C), and mediates efflux from the apical (CSF-facing) to the basolateral (blood-facing) compartment. Although FcRn involvement was not assessed in this experiment, the data are congruent with the rapid FcRn mediated brain-to-blood efflux of IgGs reported to occur across the BBB.[105] In this study, IgGs were rapidly effluxed from brain following intracerebral injection, and the efflux was saturable and inhibited by Fc fragments, but not $F(ab')_2$ fragments. Because both CSF and cerebral interstitial fluid have neutral pH, FcRn mediated IgG transcytosis requires initial uptake by

fluid-phase endocytosis, which would be favored by the large pinocytotic activity of choroidal epithelial cells (Figure 5A).

The influence of FcRn on the cerebral distribution of IgGs following intravenous injection was recently evaluated in mice deficient for the Fc receptor large subunit.[106] The pharmacokinetic model applied by the authors to IgG concentration data in blood and brain predicted a rapid brain efflux component that is not different between wild-type and FcRn deficient animals, contrasting with the apparent FcRn dependency of the efflux reported by Zhang and collaborators.[105] No statistically significant difference was observed in the low IgG levels between the two strains. CSF levels of IgGs were not measured in this study, which does not permit to conclude about the impact of choroidal FcRn on IgG distribution in the ventricular and meningeal spaces. Such information would be relevant to antibody-based therapies targeting the leptomeningial spaces, such as in CNS lymphomas, which have proved more efficient when administered intraventricularly in patients.[186] Besides its potential role in setting IgG concentration in CSF, another function of FcRn in the choroidal epithelium may be to permit endogenous CSF-borne IgG presentation to the myeloid cells that are present in the choroidal stroma and participate in neuroimmune surveillance.[187]

6. PERSPECTIVES

Progress in our knowledge about the blood−brain interfaces has ascertained the precocious establishment of their phenotype, and their crucial role in the development and protection of the brain. Both the BBB and the BCSFB serve this function, but use distinctive biochemical effectors which have been extensively characterized in small laboratory animal studies. Further work should be directed at translating these data to human, by conducting a thorough characterization of tight junction proteins, influx and efflux transporters, receptors, and enzymes. Examining the status of barrier neuroprotective mechanisms in late life stage should also deserve future attention, as defective barriers could contribute to the pathophysiology of neurological disorders during aging.

Several aspects uncovered by studies on barrier genesis have paved the way for new therapeutic strategies. In particular, the greater permeability to immunoglobulins and other macromolecules resulting from pericyte deficiency at the adult stage deserves further investigations. Deciphering the molecular mechanisms that operate at the BBB to determine opening or closure of this pathway should prove of great interest in neuropharmacology. Similarly, further elucidation of the mechanisms that support specific plasma protein transport across the choroidal epithelium in early and adult stages will provide the basis for a new pathway of drug delivery to the brain via the CSF.

The efficacy of the CP-CSF system as a route of delivery to the brain has often been denied on the basis that penetration through the CSF brain interface is too slow for the compound to build up sufficient concentrations within the tissue. This notion derives mostly from intraventricular or intracisternal bolus injections, which poorly mimic the kinetic profiles that would be generated by continuous influx across the BCSFB from a fairly constant blood compartment. Yet, this pathway, which is obviously of interest for the management of affections of the leptomeningial spaces, such as meningitis or lymphomas, should be revisited for the treatment of brain pathologies in a broader perspective.

■ ASSOCIATED CONTENT

Supporting Information

Developmental profile of LRP2 transcript levels in rat choroid plexuses. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

BBB, blood−brain barrier; BCSFB, blood−CSF barrier; CNS, central nervous system; CSF, cerebrospinal fluid; FcRn, Fc neonatal receptor; IgG, immunoglobulin G; LRP, low-density lipoprotein receptor-related protein; TfR, transferrin receptor

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