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Potential Pathways for CNS Drug Delivery Across the Blood-Cerebrospinal Fluid Barrier

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Abstract: The blood-brain interfaces restrict the cerebral bioavailability of pharmacological compounds. Various drug delivery strategies have been developed to improve drug penetration into the brain. Most strategies target the microvascular endothelium forming the blood-brain barrier proper. Targeting the blood-cerebrospinal fluid (CSF) barrier formed by the epithelium of the choroid plexuses in addition to the blood-brain barrier may offer added-value for the treatment of central nervous system diseases. For instance, targeting the CSF spaces, adjacent tissue, or the choroid plexuses themselves is of interest for the treatment of neuroinflammatory and infectious diseases, cerebral amyloid angiopathy, selected brain tumors, hydrocephalus or neurohumoral dysregulation. Selected CSF-borne materials seem to reach deep cerebral structures by mechanisms that need to be understood in the context of chronic CSF delivery. Drug delivery through both barriers can reduce CSF sink action towards parenchymal drugs. Finally, targeting the choroid plexus-CSF system can be especially relevant in the context of neonatal and pediatric diseases of the central nervous system. Transcytosis appears the most promising mechanism to target in order to improve drug delivery through brain barriers. The choroid plexus epithelium displays strong vesicular trafficking and secretory activities that deserve to be explored in the context of cerebral drug delivery. Folate transport and exosome release into the CSF, plasma protein transport, and various receptor-mediated endocytosis pathways may prove useful mechanisms to exploit for efficient drug delivery into the CSF. This calls for a clear evaluation of transcytosis mechanisms at the blood-CSF barrier, and a thorough evaluation of CSF drug delivery rates.

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1. INTRODUCTION

The fine control of the cerebral extracellular fluid homeostasis necessary for central nervous system (CNS) functions requires a strict regulation of the molecular and cellular exchanges between the blood and the brain. This regulation results from the specific and complementary properties of the blood–brain interfaces. These comprise the blood-brain barrier (BBB) proper, located at the endothelium of the cerebral microvessels, and the blood-cerebrospinal fluid barrier (BCSFB). This second interface is located at both the epithelium of the choroid plexuses and the arachnoid membrane, respectively upstream and downstream of CSF flow [1]. The CSF circulatory system participates in regulating cerebral extracellular fluid homeostasis [2]. The CSF is actively secreted by the four choroid plexuses located in the two lateral, the third and the fourth ventricles. It flows briskly across the ventricular system to reach the ventral and internal cisterns, and the subarachnoid spaces. It mixes with the fluids located in the perivascular spaces that surround the penetrating meningeal arteries and veins. It is then resorbed into the venous blood across the arachnoid villi, into the dural lymphatics through a yet unknown route across the tight arachnoid, or drains along the cranial nerves. This article briefly highlights the role of blood-brain interfaces in setting the poor cerebral bioavailability of drugs, and discusses the interest of targeting the blood-CSF barrier

and CSF compartments in addition to the BBB when designing CNS drug delivery strategies. It then describes potential strategies to improve drug delivery into the CSF and discusses the knowledge that needs to be gathered on choroid plexus functions before such strategies can be implemented.

2. THE BLOOD-CSF BARRIER IN CEREBRAL DRUG DELIVERY

The barrier-forming cells that separate the blood from the CNS are joined by a continuous belt of tight junction proteins that seal the paracellular cleft, and possess a machinery based on multispecific efflux transport proteins and detoxifying enzymes that collectively prevent the entry of potentially toxic compounds into the CNS. These specific neuroprotective functions of the blood-brain interfaces are responsible for the poor cerebral bioavailability of numerous pharmacological agents of potential interest for treating CNS diseases (reviewed in [3, 4]). A number of influx transport systems for nutrients, hormones and other biologically active molecules necessary for normal brain functions are also located at blood-brain interfaces. These transport processes involve either solute carriers (SLC) for small compounds, or receptor-mediated transcytosis pathways for larger polypeptides [1]. They represent targets of interest to develop strategies aiming at improving drug delivery to the CNS. Owing to the very large surface area developed by the BBB, the specific transport properties of this interface have been widely studied in the past decades, and several strategies have been developed to improve drug delivery across the BBB by targeting SLC transporters and receptor-mediated pathways [5].

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Subject of fewer investigations, the BCSFB located at the choroid plexuses will be the focus of this article. The best known choroid plexus function is the active and regulated secretion of CSF [6]. Choroid plexuses also are a source of bioactive compounds including trophic factors, hormones or hormone carriers necessary for brain development and maintenance [2, 7]. The implication of the choroid plexus-CSF system in neuroimmune surveillance has been recently highlighted. The choroid plexuses appear to be a preferential pathway of immune cell migration into the CSF both in physiological condition and during the early phase of neuroinflammation, and are prone to secrete immunoactive compounds under peripheral or central inflammatory stress [8-10]. In addition, a fraction of CSF and perivascular fluid-borne antigens exits the brain by draining through the dural lymphatics and along cranial nerves directly into deep cervical lymph nodes. The dural lymphatic pathway has been hypothesized to account also for immune cell exit from CSF, although cell extravasation across the tight arachnoid prior to reaching the dural lymphatics has not been investigated [11-13].

The choroid plexuses however received little attention as a potential pathway for drug delivery to the CNS. A few reports actually denied the interest of targeting the BCSFB on the basis that intracerebroventricular injection was equivalent to a slow venous infusion, because only a limited volume of brain tissue can be reached by compounds injected directly into the CSF. The argument results from the measurement of molecular diffusion from CSF into brain parenchyma, which is very slow by comparison with CSF resorption rate in the venous blood. However, the efficiency of intracerebroventricular injection mode to generate a central biological effect was also repeatedly reported for low concentrations of receptor agonists or antagonists, cytokines, neuropeptides and hormones that did not lead to detectable amount into the plasma (*e.g.* cholecystokinin, [14]). It is also intriguing that in numerous studies testing new delivery systems targeting the brain parenchyma through the BBB, efficacy of these vectorized systems is assessed by comparing the pharmacodynamic effect of the therapeutic moiety after peripheral/IV injection versus intraventricular infusion/administration. This implicitly acknowledges that the same active compound, were it delivered into CSF via the BCSFB, would be able to reach its parenchymal target. One recent example of this approach has been reported for bispecific antibodies that are antagonist of the metabotropic glutamate receptor-1 [15].

This article does not intend to discuss the interest and limitations of intracerebroventricular infusion. Targeting the BCSFB for blood-to-CNS drug delivery relates to sustained (hours) or chronic (daily doses) exposure of CSF to drugs rather than acute injection into CSF. In this context, targeting the BCSFB in addition to the BBB can have added-value in several situations and for several reasons described below.

2.1. The CSF Spaces and the Cerebral Structures Adjacent to CSF Compartments are Pharmacological Targets of Interest in Several CNS Diseases

The subarachnoid, perivascular, or periventricular spaces are areas of pathogenic lymphocyte, monocyte and neutrophil accumulation in neuroinflammatory disorders such as multiple sclerosis and related experimental autoimmune encephalitis, or virus-induced neurological disorders including neuroaids and CMV infection [16-20]. Foci of B-cells detected in different CNS autoimmune diseases and producing potentially deleterious antibodies are thought to be mainly localized in leptomeninges [21]. These fluid spaces, connected with deep cervical lymph nodes, are obvious targets for therapeutic immunoregulators. Another example is related to cerebral amyloid angiopathy inducing degenerative vascular changes. It is driven by amyloid beta (Aβ) peptide, cystatin c, transthyretin, or else gelsolin deposits around penetrating vessels [22, 23]. The

deposits are accessible through interconnected CSF/perivascular spaces [24]. A third example concerns periventricular tumors including meningiomas, pharmacoresistant ependymomas, and leptomeningeal metastases from peripheral primary tumors, all in direct contact with CSF. The blood-tumor barrier is often considered leaky, as a result of the lessened efficacy of tight junctions that allows contrast enhancement in magnetic resonance imaging. However, many anticancer drugs are lipophilic and are prevented from crossing the BBB by multidrug resistance (MDR) efflux proteins controlling the transcellular pathway, not by tight junction proteins controlling the paracellular cleft. In a number of periventricular tumors such as ependymomas, MDR proteins remain well expressed at the blood-tumor barrier [25]. Pharmacological pressure from the CSF will contribute to achieve therapeutic concentrations of drugs within the tumoral tissue.

Finally, the choroid plexuses themselves can be therapeutic targets of interest to regulate CSF production in some forms of hydrocephalus, and more prospectively to increase the secretion of biologically active factors in neurohumoral dysregulation and perinatal developmental diseases, or to control neuroimmune interactions in CNS diseases with a neuroinflammatory component. In these cases, the drugs do not have to undergo complete transcytosis to reach the CSF.

2.2. The CSF as a Significant Pathway Towards Deep Structures of the Brain Remains to be Explored in Detail in the Context of Chronic Drug Exposure

Previously seen as a simple passive drainage system for the brain, the CSF circulation is increasingly recognized as participating in volume transmission for polypeptides, vitamins, and hormones. For instance, transthyretin specifically secreted by the choroid plexus contributes to maintain thyroid hormone homeostasis throughout the brain. Other hormone carriers, growth and differentiation factors, guidance molecules and neuroprotective factors are produced by the CP and have a central action during development and throughout life [2]. Diffusion/movement of CSF-borne material into deeper cerebral structures is favored by the relatively high ratio of the ependymal/*glia limitans* surface area to the volume of adjacent CSF, by the tortuous and compartmentalized CSF flow pathway, and by the relatively slow rate of CSF flow. In rat, 80% of a polar material of moderate size present in the lateral ventricle will be lost from CSF by diffusion in periventricular structures before it reaches the cisterna magna [26, 27]. The *glia limitans* situated downstream of the cisterna magna is thick in places and may act as a reservoir for CSF-borne compounds [26]. Routes from CSF to deeper structures may also involve the perivascular spaces surrounding the penetrating arteries and veins that are interconnected with subarachnoid and cisternal CSF. While these flow pathways are likely of limited relevance in the context of short-term intracerebroventricular injection, they may be efficient for slow continuous circulation of biologically endogenous compounds. Brain pulsation resulting from cardiac beats, and a possible fluid movement between parenchymal arterial and venous perivascular spaces, somehow involving the glial aquaporin-4 [24, 28, 29], can favor dissemination through these pathways. Finally, axonal transport from periventricular tissue to deep cerebral structure has been demonstrated by MRI in both rat and primate, at least for ions such as manganese [30, 31].

Studies combining long-term chronic treatment with brain mapping in view to evaluate to what extent drugs are delivered to deep structures through these pathways are lacking, but it is conceivable that small or large pharmacological compounds that may continuously reach the ventricular CSF through the choroid plexus pathway, follow routes that are similar to those used by CSF-borne endogenous compounds.

2.3. Increasing Drug Concentration into the CSF will Reduce the CSF Sink Action Toward Drugs that Reached the Brain Parenchyma Through the Blood-Brain Barrier, thus Contributing to its Sustained Extracellular Fluid Concentration

Among pharmacokinetic factors that set the cerebral bioavailability of a drug, and hence its pharmacological activity, both the rate of transfer from blood to brain, and the rate of elimination from brain are important. Regarding the latter, cerebral drainage and CSF sink action significantly contribute to the cerebral elimination of polar compounds that are delivered via unidirectional drug delivery systems at the BBB. Targeting transcytotic mechanisms that are present and efficient at both barriers will offer the advantage of raising the drug concentration in CSF and thereby limiting the sink action towards compounds delivered through the BBB. However, little is known on the similarities and differences in endocytic and transcytotic pathways and mechanisms between the BBB and the BCSFB (See Part 3).

2.4. The Blood-CSF Barrier is of Particular Relevance in the Context of Neonatal and Pediatric Pharmacology

The previously prevailing hypothesis that the blood-brain interfaces of the developing brain are immature, and therefore "leaky" is now challenged. A continuous belt of tight junctions is formed early during prenatal development at both the blood-brain and blood-CSF barriers in rodent as in human, and the paracellular cleft between adjacent cells of the blood-brain interfaces is sealed to polar tracers [32-36]. In rat, the barrier phenotype is acquired throughout the entire CNS before birth, a stage that can be compared to midgestation in humans. Blood-brain interfaces regulate the composition of brain fluids during development as in adulthood, albeit by mechanisms that differ with age [3, 33, 37, 38]. While this early control of the CSF composition relative to plasma composition is a prerequisite for the CSF to fulfill its function in brain maturation, it infers that blood-brain interfaces need to be overcome to allow efficient drug delivery to the CNS during the peri/post-natal period as in adults.

The capillary density and the cerebral blood flow during pre- and post-natal development are both lower than in adult [39, 40]. In contrast, the choroid plexuses develop early during development. Choroidal blood flow has not been measured in peri/post-natal stages, but the choroid plexuses appear already richly vascularized. This emphasizes the importance of the choroid plexuses relative to the cerebral blood vessels as a route of entry into the CNS at perinatal stages of development. The total volume of CSF relative to the brain size is also much higher in developing animals than in the adult. The CSF-to-cranial cavity volume ratio is 28% at E19 and 20% at P2 in rat [36]. This factor, combined with a low CSF turnover compared to the adult, would further favor the access to cerebral structures for therapeutic agents delivered into the CSF. In line with this, the apparent blood-to- brain parenchyma permeability of selected tracers in developing animals appears to result mainly from their brain entry through the choroid plexus-CSF route [33, 38]. This is also relevant to the critical role of the choroid plexus-CSF system in delivering trophic factors and guidance molecules to nerve cells and their precursors during brain development and maturation [41].

Overall these different elements highlight the importance of not underestimating the blood-CSF barrier when considering strategies to deliver drugs to the brain. The pertinence of specific carrier proteins that can be targeted for CSF delivery of drugs, including that of large molecules, has not been extensively analyzed. The next chapter reviews potential strategies to deliver drugs to the CSF across the choroid plexuses, describes known properties of choroidal proteins seemingly involved in transport or receptor-mediated endocytosis at the choroidal epithelium, and points to the shortcoming in the knowledge of transcytosis mechanisms at the choroidal

epithelium that need to be filled in view to optimize drug delivery to the brain.

3. ROUTES OF DRUG DELIVERY INTO THE CSF THROUGH THE CHOROID PLEXUS

3.1. Anatomical Considerations

Choroid plexuses are highly branched structures organized in fronds, each of those forming several villi. The stromal core of the tissue, surrounded by the BCSFB-forming tight epithelium is a loose and densely vascularized connective tissue, with vessels extending to the tip of each villus (Fig. **1A,B**) [42]. The choroidal vessels are large non-sinusoidal fenestrated capillaries with diaphragmed fenestrae, similar to those of many peripheral organs, and exert no restrictive effect on plasma solute and protein transfer [1]. The physiological upper limit of the pore size in choroidal vessels, determined by the open space within the diaphragmed fenestrae, is close to 12 nm, which represents the diameter of very large proteins such as ferritin [43]. Accordingly, immunostaining of various plasma proteins or direct observation of ferritin by electron microscopy demonstrated their ability to extravasate into the stromal compartment of choroid plexus [44, 45]. In addition to these fenestrae, Coomber and Stewart [46] described single vesicle-forming transendothelial channels in regions of attenuated cytoplasm in choroidal vessels. These channels displayed a large aperture around 50 to 60 nm. While this pore size is compatible with a free diffusion of lipoproteins, small metallic or polymeric nanoparticles, it is unlikely that liposomes (size in the 100 nm range) will equilibrate freely between plasma and the choroidal stroma.

3.2. Pathways of Drug Delivery Across Brain Barriers. What Does Apply to the BCSFB?

In non-pathological conditions, intercellular diffusion via the paracellular cleft is by essence restricted in these interfaces by occluding tight junctions. Strategies have been developed to reversibly open this route across the BBB. Osmotic disruption with hypertonic solutions is being used in brain chemotherapy [47]. More recently, low power focused ultrasound technologies, combined with intravascular microbubbles has demonstrated its efficacy in delivering drugs to localized areas of the brain in animals, offering a possible noninvasive alternative [48]. These approaches, as well as invasive methods that bypass the barriers by direct intraparenchymal, intracerebroventricular, or intralumbar administration have been reviewed elsewhere [5, 49]. Possible effects of osmotic disruption on the permeability of the BCSFB have not been reported.

Transcellular pathways of conventional drug delivery involve one of the following mechanisms: simple passive diffusion, facilitated diffusion by membrane carriers, or vesicular transfer.

3.2.1. Passive Diffusion and Influence of Efflux Mechanisms

The rate of transfer of a drug by passive diffusion will be set in part by two major physicochemical features, size and lipid solubility, the latter being influenced by the ionization state of the molecule at physiological pH. This mechanism offers no specificity for the targeted tissue and transfer is determined by the free drug concentration gradient. Many conventional neuroactive drug candidates designed to fulfill adequate criteria (size below 500 Da and sufficient lipid solubility) yet do not achieve efficient therapeutically relevant concentrations in the brain. One of the reasons is that brain barriers possess a large number of efflux transporters of broad specificity that exclude these molecules from the brain. These transporters are distributed between the two membrane domains of the polarized barrier cells and can act from either pole to influence the drug concentration in the targeted tissue. They belong to 2 large families, the outwardly-directed energy-dependent ATP binding cassette transporters (ABC), and the solute carriers (SLC) [1]. The BBB and the BCSFB display distinct panels of efflux transport systems, which may be relevant to specific endogenous substrates
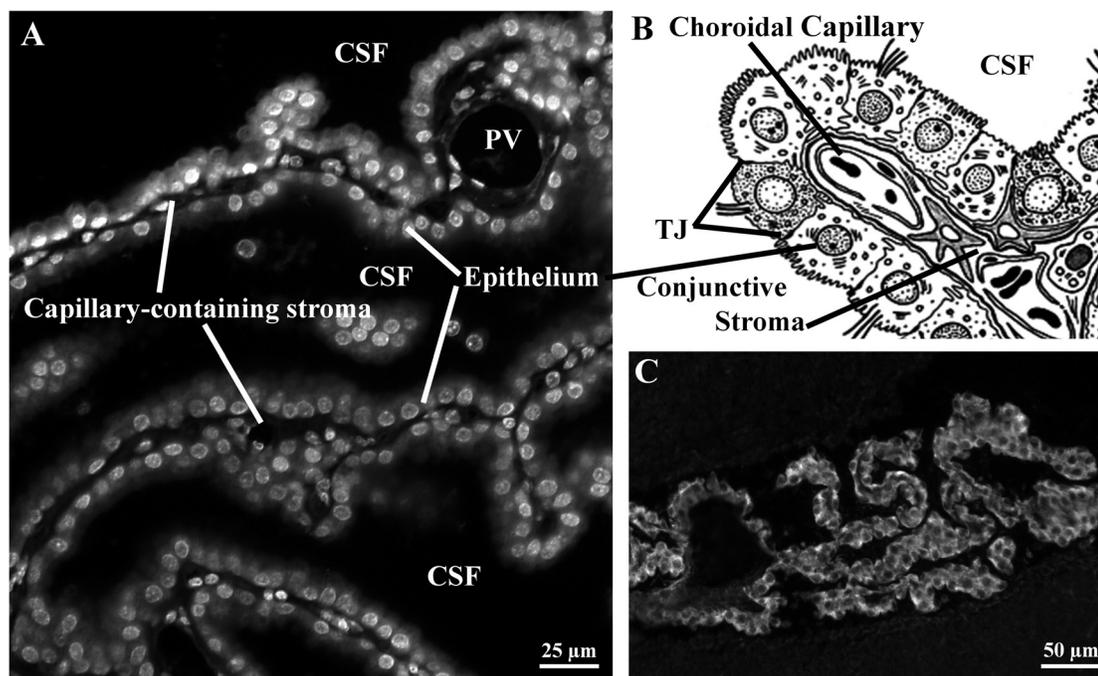
Fig. (1). The blood-CSF barrier.
A and B: Paraffin section from non-human primate choroid plexus with dapi nuclei staining (A) and schematic drawing (B) of choroid plexus villi showing the choroidal epithelium facing the CSF and delimiting the stroma filled with loose conjunctive tissue and fenestrated capillaries. PV: larger penetrating vessel. TJ: tight junctions. C: Frozen section of rat brain tissue showing a strong LRP1 immunolabeling associated with the choroidal epithelium. Antibody #3501 from American Diagnostica Inc was used following paraformaldehyde fixation. No signal was observed in absence of primary antibody.

and/or to the nature of the respective environment of each barrier [50]. The role of ABCB1 and ABCG2, both hallmark transporters of the BBB luminal membrane has been extensively studied, and these transporters are regarded as being the major factors responsible for the poor cerebral penetration of many anticancer drugs. Similarly, efflux transporters at the BCSFB affect drug penetration/concentration in the CSF and in brain to a substantial and therapeutically significant extent. Evidence for that has been reported some decades ago for β-lactam antibiotics, well before the molecular identity of choroidal efflux systems was established. Pharmacokinetic parameters for benzylpenicillin and imipenem, which have very different CSF disposition, were compared [51]. The unidirectional influx determined by the in situ perfusion method was similar for both compounds, whereas the CSF-to-plasma unbound concentration ratios at pseudo steady-state was 13-fold higher for imipenem. Clearance from the CSF examined after intracerebroventricular administration showed that benzylpenicillin was cleared much more rapidly than imipenem and mannitol, and that the clearance process was probenecid sensitive. Collectively, these data suggested that an active efflux of benzylpenicillin across the BCSFB was responsible for its low CSF disposition and its poor therapeutic efficacy in the treatment of CNS bacterial infections. A definite answer to the question has been provided with the availability of knockout animals for certain choroidal transporters. As mentioned, the BCSFB has a partially different set of efflux proteins. It shares with the BBB, the expression of ABCC4/Mrp4, which locates at the basolateral blood-facing membrane. By contrast, it selectively expresses the proton-coupled oligopeptide transporter SLC15A2/Pept2, present on the apical CSF-facing membrane. The impact of the latter on the brain exposure of the antibiotic cefadroxil was demonstrated in knockout mice [52]. The CSF-to-blood concentration ratio was 6- to 7-fold higher in SLC15A2 deficient animals compared to wild type, while the cortex-to-CSF concentration ratio was 3-fold lower. These results indicated that SLC15A2-mediated clearance of the compound across the choroid plexus contributes to the poor efficacy of this antibiotic in the

treatment of CNS infection. This efflux at the BCSFB is likely to impact the cerebral penetration of many SLC15A2 substrates, such as other aminocephalosporins, or the antiviral agent valacyclovir. The impact of ABCC4 was evaluated toward the penetration of the antitumoral drug topotecan [53]. Interpretation is more complex because of the transporter expression at both barriers. Yet, concentrations of the drug measured in CSF by ventricular microdialysis showed a 10-fold ratio between wildtype and deficient mice over a period of 3 hrs. The fact that this strong difference was already noticeable within the first 15 minutes following topotecan intravenous administration clearly indicated that choroidal ABCC4 substantially limits the CSF concentration of its substrates. Brain concentrations of topotecan measured 2 hours after intravenous injection were also 6-fold higher in the deficient mice compared to the wildtype, but were not different at the earlier time-point of 15 min. This actually suggests that ABCC4 mediated efflux at the BCSFB is the actual primary factor influencing CSF and brain disposition of topotecan. Given the significant impact of efflux transporters at the BCSFB, the affinity of CNS drug candidates for these systems should be taken into account, especially in the case of anti-infectious agents or anticancer drugs intended for the treatment of infectious meningitis or periventricular and meningeal tumors, respectively.

3.2.2. Carrier-Mediated Influx Transporters

In order to enhance the diffusion of small therapeutic compounds into brain, drug development has targeted influx mechanisms that are physiologically active in barrier cells where they serve the crucial purpose of supplying indispensable nutrients to the brain. Glucose, amino acids, nucleosides, monocarboxylate, some vitamins, *etc.*, are small lipid insoluble molecules that require carrier proteins to diffuse across cell membranes. These carriers are members of several SLC subfamilies [2], and function through energy-dependent or facilitative processes. They are required at both membrane domains of the cells to enable full vectorial transport from blood to brain. Although the expression of these carriers

is not restricted to brain barrier cells, this approach was expected to substantially favor selective cerebral delivery compared to non-specific passive diffusion. The nutrient carriers have a narrower substrate specificity than efflux transporters, which warrants only minor structural and physicochemical modifications on the drug candidate in comparison to the endogenous substrates. Another drawback of this hijacking strategy is the obvious competition arising between the transported drugs and the native substrate. Given all these limitations, this approach has been relatively unsuccessful, and among CNS drugs that are currently used in the clinics, only few of them are transported by facilitative carriers ([5, 54] for extensive lists). The best characterized example is that of the anti-parkinsonian drug L-DOPA, whose penetration in brain is favored by the large neutral amino acid transporter LAT1 (SLC7A5/SLC3A2). This carrier also contributes to the cerebral penetration of antiepileptic compounds gabapentin and pregabalin. Another antiepileptic drug, valproic acid, as well as its derivatives, is transported by the monocarboxylate transporter MCT1/SLC16A1. Several drugs approved for the treatment of Alzheimer's disease such as the cholinesterase inhibitor donepezil or the N-methyl-D- aspartate receptor antagonist memantine are substrates for polyspecific cation tranporters of the SLC22 subfamily. In vivo brain perfusion studies using prototypical inhibitors suggested that transport mediated by OCTN1/SLC22A4 and OCTN2/ SLC22A5 respectively contribute along with passive diffusion to the entry of memantine and donepezil in CNS [55, 56]. The role of the influx transporters active at the BSCFB has not been considered. Yet, LAT1 like MCT1 have been identified in the rat choroidal epithelium by immunohistochemistry [57, 58], and transcripts of OCTN1 and OCTN2 genes have been detected in this interface. As they all mediate facilitated diffusion of their substrates, the flux directionality set by the concentration gradient is expected to be similar at both barriers. One can reasonably postulate that, unless saturated by an endogenous substrate, influx transporters of the BCSFB (path 1 in Fig. **2**) will work in concert with those of the BBB to build up the intracerebral concentration of a drug substrate.

3.2.3. Transcytosis Pathways

Finally, transcellular transfer across barriers can proceed by transcytosis ((paths 2-4 in Fig. **2**). This is the obligatory pathway for large molecules such as polypeptidic hormones, metal carriers, lipoproteins that are required for normal brain functions. Transcytosis sequentially involves endocytosis, i.e. internalization of an extracellular cargo into vesicles that invaginate from the plasmalemma at one front of the cell, sorting of the cargo and trafficking of the vesicle in the endosomal network across the cell, fusion of the vesicle membrane with the target membrane at the opposite front, and release of the vesicular content in the extracellular space. Endocytosis is an energy-dependent process involving one of the following mechanisms. Vesicles can form from distinct areas of the plasmalemma named clathrin-coated pits. These pits are enriched in various cell surface receptors, whose binding by their ligand triggers the endocytic event. Other vesicles originate from caveolin-1-containing lipid rafts, another type of specialized membrane invagination also called caveolae. These vesicles are also engaged in receptor-mediated transcytosis, as well as in fluid phase and adsorptive endocytosis. Fluid-phase endocytosis mediates the random uptake of extracellular fluid and all the solutes it contains, while adsorptive endocytosis is triggered by electrical interactions between negative charges of the glycocalix at the cell plasma membrane and positive charges on the cargo. A third type of vesicles forms from non-clathrin-, non-caveolin lipid rafts (see [59-61] for detailed reviews on these different mechanisms).

Of note early works by electron microscopy have suggested that vesicular density is very low in the cerebral endothelium compared to other endothelia and that fluid-phase endocytosis tracers such as horseradish peroxidase are not transcytosed in the parenchyma in non-pathological conditions [62]. Pericytes have recently been identified as key regulators of BBB permeability, by down regulating a non-specific transcytotic mechanism possibly initiated by fluid-phase endocytosis as it did not discriminate between albumin, horseradish peroxidase or polar 70 kDa dextran [63]. The pericyte-induced downregulation of transcytosis may result, at least partially, from redirection of endosomes to the lysosomal compartment for degradation [64]. By contrast, in choroidal epithelial cells, electron microscopy consistently shows a high density of vesicles including both clathrin-coated and non clathrin-coated vesicles forming from or in close proximity to both the apical and basolateral membrane domains of the cells, suggestive of a strong endocytotic activity in the BCSFB [1, 65]. Functional annotation of highly expressed gene sub-datasets in human and mouse laser microdissected choroidal epithelium cells yielded several significant canonical pathways [66]. Among those are the "clathrin mediated endocytosis signaling" and the "caveolin mediated endocytosis signaling" pathways. This strong endocytotic activity is believed to pertain to the vigorous metabolic and synthetic functions of the BCSFB. The exact relevance of this vesicular abundance to the transcellular transport of selected endogenous or therapeutic macromolecules between blood and CSF in either direction remains completely unknown, at least for fluid-phase endocytosis as investigated with horseradish peroxidase [67, 68].
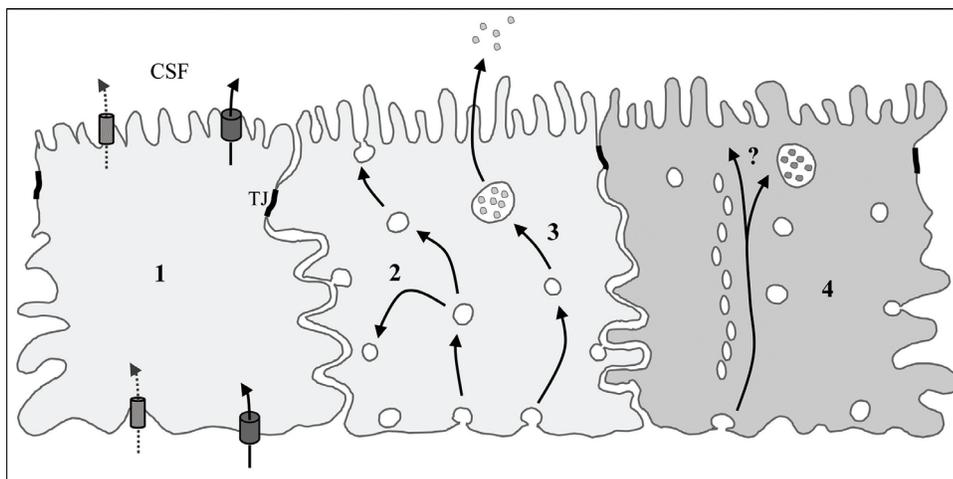
Fig. (2). Putative transport pathways for drug delivery across the BCSFB.
1. Facilitated transport involving carriers at both membrane domains; 2. Receptor-mediated transcytosis with exocytosis of the sorted cargo in CSF and receptor recycling; 3. Receptor-mediated endocytosis followed by sorting in multivesicular bodies and exosome release in CSF; 4. Plasma protein transport in specific cells, involving a transcellular tubular system and possibly multivesicular bodies. See text for detailed description of these pathways. TJ: tight junctions.

Transcytosis pathways have raised considerable interest in the field of CNS delivery for their potential to deliver large cargoes including biotherapeutic agents, nanobodies, liposomes, nanoparticles, as well as vectorized forms of these nanotechnologies-based systems. Cationization approaches have been developed in order to promote adsorptive mediated endocytosis of therapeutic agents [5]. Cationic cell penetrating peptides in particular have been investigated because of their established potential to deliver cargoes across membranes, although the internalization mechanisms remain partially elusive [69]. They can be naturally occurring peptides or fragments of proteins, such as the HIV transactivating factor Tat, the Drosophila transcription factor antennapedia alpha helix domain (penetratin), or fragments of the bacterial protegrin (Syn peptides). Conflicting results have been reported concerning the enhanced brain penetration of conjugated drugs in comparison to the unconjugated molecule (reviewed in [70] for Tat conjugates). In addition to stability and toxicity issues inherent to cell penetrating peptides, the lack of selectivity of adsorption-mediated endocytosis imparts little potential to the cationization approach for brain delivery.

Receptor-mediated transcytosis implicitly enables organ selectivity, providing the receptor is highly expressed in the targeted barrier cells. In this approach, the therapeutic entity (small chemical, protein or nanoparticle/liposome) is conjugated to a vector, whose binding to the receptor elicits the endocytotic internalization of the complex. Different types of vectors have been explored. Using the native ligand or a fragment of the ligand that retains the binding capacity will invariably interfere with the brain uptake of endogenous molecules or with physiological processes when the receptor is also endowed with a signaling activity. Antibodies directed against the receptor have been developed as an alternative, but require to be carefully optimized regarding their affinity for the receptor, as this was shown to critically impact the intracellular trafficking of the endocytosed complex [71-73]. More recently, panning technologies were applied to search for nanobodies or small noncompetitive peptide ligands using phage or yeast display libraries. The latter peptides unrelated to the native ligand will usually not compete for binding with the endogenous substrate. Like for antibodies, the binding affinity needs to be carefully tuned by chemical modifications [74].

The next part of this review will focus on the receptor-mediated transcytosis pathways that have been extensively studied as means to enhance CNS drug delivery across the BBB. It will briefly describe the underlying mechanism and review our current state of knowledge concerning these pathways at the BCSFB, based on gene expression studies and protein immunoreactivity data. When available, functional evidence of their efficacy will be discussed. Finally, emergent BSCFB specific pathways will be presented and discussed. These different pathways are summarized in Table **1**.

3.3. Receptor Mediated Transcytosis Through the BCSFB

3.3.1. Transcytosis Mechanisms Common to the BBB and BCSFB

3.3.1.1. The Transferrin Receptor Pathway

The transferrin receptor TfR1 has been one of the first receptors targeted to enhance brain delivery of compounds, because of its selective expression in cerebral microvessel endothelium compared to other endothelia. TfR1 is also expressed in the BCSFB and has been identified in rat and human choroid plexus epithelium [75-77]. The pathway of TfR1-mediated iron delivery into cells has been well characterized. Iron-loaded transferrin binds to TfR1 in clathrin-coated pits at the cell membrane, and the complex is internalized by endocytosis. Iron is released from transferrin in acidifying endosomal vesicles and exported to the cytosol for further use in basic metabolic cellular functions or for storage. TfR1 is recycled to the cell membrane where it releases iron-free transferrin whose affinity for the receptor is low at neutral pH. Delivery of iron to the brain by this route thus requires the export of cytosolic iron at brain facing membranes of the BBB and BSCFB cells, which could involve ferroportin or another mechanism still unknown [78]. Alternatively, iron supply to the brain could involve transcytosis of the ligand receptor complex and the release of iron-bound transferrin, although it is not clear why and how the high affinity holo-transferrin would dissociate from TfR1. Early works have shown that iodinated transferrin is transcytosed by a TfR1-dependent vesicular mechanism, although it was not believed to be the primary route for iron entry into the brain [58, 79, 80]. The demonstration that a monoclonal anti-TfR antibody OX-26 as well as an OX-26-methotrexate conjugate were transported across the BBB and that it resulted in enhanced cerebral delivery of the drug [81], led to ex-

Table 1.   Endo/transcytosis pathways identified at the blood-CSF barrier with a potential interest for implementing cerebral drug delivery strategies. (see text for details and references).

Receptor	Endogenous Substrate	Evidence of Transcytosis Across Choroid Plexus
Transferrin Receptor TfR1	Transferrin	Indirect
Insulin Receptor IR	Insulin	No data
LDL receptor	LDL	No data
Lipoprotein receptor-related protein LRP1	Various (α2-macroglobulin, matrix metalloproteases, different carrier proteins…)	Indirect
Lipoprotein receptor-related protein LRP2	Partial overlapping substrate specificity with LRP1, Morphogenic factors, leptin, insulin-like growth factor I (developmental relevance)	Yes, for endogenous substrate
Lipoprotein receptor-related protein LRP8	Partial overlapping substrate specificity with LRP1, selenoprotein P	Partial, indirect
Folate Receptor FRα	Folate	Yes, for endogenous substrate, through exosome
Unknown (possibly SPARC, Glycophorins)	Plasma proteins (albumin, fetuin, hemopexin, α-fetoprotein)	Yes, for exogenous proteins

tensive work aiming at designing and optimizing engineered antibody-based shuttles for small chemotherapeutic agents and macromolecules [73]. The affinity of the antibody for the targeted receptor is a particularly sensitive criterion to be considered, as it controls the trafficking of endocytic vesicles and reroutes them to lysosomes [71].

As for the BBB, iron delivery across the BCSFB is thought to occur quantitatively through the classical endocytotic TfR1-dependent route, followed by iron release in the cytosol and export in the CSF, rather than by transcytosis. Yet, recent evidence supporting that TfR1-mediated transcytosis occurs in the choroidal epithelium has been reported using a novel engineered receptor/ligand system specifically expressed in this barrier [82]. Mouse were intraventricularly injected with a AAV9 vector expressing the C terminal part of the human transferrin receptor fused to cohesin 7, a bacterial protein involved with its partner dockerin in cellulosome assembly. Cohesin 7 binds with high affinity and specificity to dockerin, which was expressed in this study as a 37 kDa fusion protein with GFP, and used as a ligand to investigate the transport capacity of the system. GFP-dockerin injected intravenously labeled a moderate number of epithelial cells in all choroid plexuses along the ventricular system. Confocal microscopy indicated that the fluorescent signal was associated with both basolateral and apical membrane domains, and with the cytosol. Electron microscopy examination of GFP-dockerin immunolocalized with an electron dense substrate also identified particles within the apical microvilli. The mechanism remains elusive, in particular the nature of the event triggering endocytosis, possibly involving binding of endogenous transferrin, and the vesicular pathway followed by GFP-dockerin. Given the very high avidity of cohesin-7 for dockerin, it is likely that the human TfR1 was sorted with its ligand and reached the apical membrane, offering a mechanistic basis for evaluating whether TfR1-mediated transcytosis at the BCSFB also impacts the cerebral penetration of antibody-based therapeutic drugs developed to target the BBB. Hint of this impact was provided in the course of evaluating the delivery and efficacy of bispecific antibodies targeting both the TfR1 for BBB crossing and the β-secretase BACE1, an enzyme with an extracellular catalytic domain responsible for Aβ production from the amyloid precursor protein. Choroid plexuses produce the precursor protein, express BACE1 and have a β-secretase activity. Amyloid β immunoreactivity was observed at the apical membrane of the choroidal epithelium [83, 84], and secretion of the peptide by the BCSFB is believed to contribute to CSF levels of Aβ and to the overall Aβ burden in the brain. Acute peripheral administration of the bispecific humanized TfR1/BACE1 antibody in non-human primate decreased Aβ levels by 20% in the cortex after 24 hours [85]. CSF levels of Aβ were reduced to a much greater extent of 50% in the same period. Delivery of the therapeutic antibody through the BCSFB via the TfR1 pathway would obviously contribute to this differential pharmacodynamic effect between both brain compartments, but was not examined in the study.

The canonical pathway in which TfR1 is endocytosed and recycled could also be exploited in CNS delivery strategies, as reported for gold nanoparticles [86]. These particles were attached to the native ligand transferrin by a pH sensitive linker, thereby mimicking the type of binding existing between iron and transferrin. The targeted particles showed increased brain penetration following systemic administration in mice, compared to nanoparticles bound to transferrin by a non-cleavable linker. These data suggest that acidified endosomal pH causes the dissociation of the gold particle from the stable complex transferrin-TfR1, and facilitate its access to brain parenchyma. The sorting process remains to be examined, to determine whether it enables normal TfR1 recycling and to identify the mechanism by which particles are released at the endothelial abluminal membrane. This drug delivery strategy, which utilizes the canonical endocytosis pathway definitely active in the choroidal epithelium, deserves to be investigated at the BCSFB.

3.3.1.2. The Insulin Receptor Pathway

Transport of insulin across the BBB occurs via a receptor-mediated transcytosis mechanism [87]. Based on the paradigm of the OX-26 anti-TfR1 antibody, a monoclonal antibody was produced against the human insulin receptor. It was found to be endocytosed in human cerebral capillaries and to undergo rapid transcytosis in the brain parenchyma of non-human primates [88]. This has led to the development of an antibody-based delivery platform, which can be used to engineer recombinant bifunctional fusion proteins and deliver therapeutic proteins such as growth factors or enzymes across the BBB [89]. Fusion constructs of a humanized version of the anti-insulin receptor monoclonal antibody with the lysosomal enzymes alpha-L-iduronidase or iduronidate-2-sulfatase are currently evaluated in phase I clinical trial in patients with two lysosomal storage disorders affecting the brain, Hurler's syndrome and Hunter's syndrome [90].

Early studies that investigated the distribution of insulin receptor in the brain reported that, in addition to the BBB, choroid plexuses also possessed a high insulin binding capacity [91, 92]. Computerized densitometric analyses indicated that choroid plexus actually displayed the highest density of insulin binding sites among all brain structures [92, 93]. In situ hybridization confirmed the expression level of the insulin receptor gene in choroid plexus [94]. Direct evidence that the insulin receptor mediates insulin transcytosis across the BCSFB has not been reported to our knowledge. Studies in dog and human indicated that continuous blood infusion of insulin raised the CSF level of the hormone concurrently to the plasma level [95, 96]. Modeling of the kinetics of insulin uptake in CSF from plasma suggested the presence of an intermediate compartment between blood and CSF, but did not provide a clear answer as to the nature of this compartment [95, 97]. It could correspond to the parenchymal interstitial fluid, or the choroid plexus tissue, or both.

Rapid elimination of insulin from the CSF following intracerebroventricular perfusion, relative to the elimination of a CSF bulk flow marker suggested that insulin receptors in the BCSFB could mediate CSF-to blood transcytosis of the hormone, thus contributing to insulin signal termination [98]. Assessment of the subcellular distribution of the receptor in the choroidal epithelium and of the directionality of a transcytotic process, if it occurs, is needed to appreciate the relevance of this pathway in drug delivery strategies. Given the abundance of insulin receptors in choroid plexus, it would be of great interest to determine whether insulin receptor-mediated transcytosis enhances the cerebral delivery of antibody based fusion therapeutic agents across the BCSFB.

3.3.1.3. The Low Density Lipoprotein Receptor Pathway

The low density lipoprotein (LDL) receptor is a high affinity receptor involved in cell membrane cholesterol homeostasis. It binds cholesterol-carrying LDL particles via the apolipoprotein B moiety, and mediates their endocytosis through coated pits. The LDL particles are delivered to lysosomes for degradation and cholesterol release, while the receptor is recycled to the cell surface. The LDL receptor is expressed at the BBB in higher amounts than in other endothelia and serves a function of delivering cholesterol to brains cells by mediating the transcytosis of LDL particles [99]. Peptide ligands binding to the extracellular domain of the human LDL receptor have been identified by phage display biopanning [74]. Chemical optimization yielded a peptide that presents a high affinity for the receptor and is not competitive for endogenous LDL. Biphoton microscopy imaging *in vivo* demonstrated that this peptide can extravasate from spinal cord vessels and accumulate in the perivascular parenchyma, while a scrambled version of it remained sequestered in the vessel lumen. Further investigations are needed to characterize the transcytosis mechanism and evaluate its efficacy to deliver a therapeutic cargo into brain. Little is known

concerning the expression of the LDL receptor at the BCSFB. Transcripts have been detected in mouse choroid plexus [100] . A recent immunohistochemical analysis of the receptor in human choroid plexus showed consistent positive signal in choroidal epithelial cells across the whole series of tissues collected from seven patients [101]. Future studies evaluating the potential of LDL receptor-mediated transcytosis in CNS drug penetration should investigate in parallel the BBB and the BSCFB.

2.3.1.4. The LDL Receptor-Related Protein Family

LDL receptor-related proteins (LRP) form a family of cell surface receptors, all structurally related to the LDL receptor [102]. LRPs act as receptors for macromolecular ligands, which are internalized in clathrin-coated pits and directed to lysosomes for degradation. Ligands include protease/protease inhibitors complexes, as well as loaded carrier proteins for hormones and vitamins, such as thyroglobulin, transthyretin, sex-hormone binding protein, retinol binding protein, or vitamin D binding protein [102, 103]. LRPs can also mediate transcellular delivery of their cargo across barrier-forming cell [104]. Melanotransferrin was shown to accumulate substantially in the mouse brain following intravenous injection or brain perfusion, by a mechanism competed by LRP ligands [105]. LRP1 (also designated as LRP or α2-macroglobulin receptor) was presumed to be responsible for the process and appeared as a novel receptor to target in order to improve cerebral drug delivery. Based on the observation that apoprotin and several other LRP1 ligands all possess a Kunitz protease inhibitor domain, peptides derived from the human sequence of this domain were synthesized and screened for their CNS penetrating potency [106]. One of them, designated as angiopep2, was selected for its high volume of distribution in mouse brain parenchyma. Initial brain uptake of the labeled compound in the mouse brain as well as luminal-to-abluminal transfer across an in vitro BBB model were inhibited by known LRP1 ligands or by angiopep2 itself [106, 107]. The inhibition was only partial, indicating that angiopep2 brain uptake involves both LRP1-mediated transcytosis and a LRP1-independent non saturable component. Angiopep2 has been developed as a vector platform for brain delivery. ANG1005, a angiopep2-conjugated derivative of paclitaxel, is currently in clinical phase II for the treatment of patients suffering from breast cancer with recurrent brain metastases [108], or patients with recurrent high-grade glioma [109] .

Despite the development of the angiopep platform as a LRP1-targeting brain delivery vector, and numerous functional studies implicating LRP1 in the brain penetration or brain clearance of macromolecules across the BBB, the actual presence of this receptor in the brain microvascular endothelium remains a matter of debate. The receptor was immunodetected in mouse brain microvessels [110]. Conversely, LRP1 could not be detected at the BBB in human brain tissues [111, 112], or was detected in only two brain samples out of seven in a recent study on human tissue [101]. In chicken, only brain penetrating large vessels were mentioned as positively stained [113]. In agreement with this last set of data, LRP1 was not detected in the proteome of CD31^{+}/CD45^{-} endothelial cells from mouse brain [15]. Following these observations, it was proposed that LRP1 cargoes actually get access to the brain across the BCSFB [114]. LRP1 has been immunolocalized more consistently in rat, chicken, and human choroid plexus, in which the signal apparently distributes at both apical and basolateral membranes, and in the cytosol of epithelial cells (Fig. **1C**, and [101, 113, 115]). LRP1 immunosignal in rat was described as very intense on the choroid plexus epithelium, compared to peripheral organs or other cerebral structures [115]. Direct evidence for LRP1-mediated endocytosis or transcytosis at the BCSFB is lacking. Many ligands for this receptor have been identified in CSF, some of them likely secreted by the choroid plexus, such as α2-macroglobulin or matrix metalloproteases 2 and 9 [102, 103, 116] Presumably, LRP1 in choroidal cells would mediate CSF clearance of protease/α2-macroglobulin complexes from the CSF, and participate in protease

activity homeostasis in brains fluids. However, its function at the BCSFB should be reconsidered, and its possible contribution in the brain delivery of angiopep2-based drugs needs to be evaluated.

Strategies to target other LRP members for CNS delivery have not been implemented. Two other LRPs represent potential candidates of interest, because of their substantial expression level in the BCSFB. LRP2/megalin plays a crucial role in brain patterning by controlling concentrations of morphogenic factors in brain fluids during early embryogenesis, and later on by providing the subependymal stem cell niche with a microenvironment enabling adult neurogenesis [117, 118]. This control involves receptor-mediated endocytosis of LRP2 ligands and their subsequent degradation. Accordingly, LRP2 is highly expressed in the neuroepithelium and remains present only in a restricted domain of the lateral ventricle ependyma in the adult. LRP2 is also expressed in choroid plexus, with a similar developmental pattern. A quantitative analysis of LRP2 gene expression in rat choroid plexus showed high levels of transcripts in the perinatal period (at embryonic day 19 and postnatal day 2), which substantially decreased at the adult stage [1]. LRP2 immunoreactivity was observed at the apical membrane of choroid plexus epithelial cells in 3-day-old rats [119]. The receptor was also immunodetected in the adult mouse, rat, and human choroid plexus, where it stained the whole epithelial cell [101, 120]. Functional studies in adult rats presenting a partial and localized LRP2 deficiency at the BCSFB following intraventricular injection of a lentiviral silencing vector have indicated that several LRP2 ligands are transcytosed from blood to CSF across the BCSFB [1]. Leptin [121] and the insulin-like growth factor I [120] were shown to accumulate in CSF to a lower extent in the deficient animals compared to control rats. All together, these data point out that LRP2 could serve as a receptor /transporter for therapeutic cargoes, provided an efficient carrier ligand can be identified. Peptide based drug delivery systems targeting LRP2 are currently developed to treat renal disorders. Such kidney-specific carriers however may not be optimized for cerebral delivery if they follow the canonical endocytic pathway of LRP2 in proximal tubular cells, which ends up in the lysosomal compartment.

A third member of the LRP receptor family, LRP8/ApoER2, may represent a good candidate to target for improved delivery to the CSF. *In situ* hybridization has shown high levels of LRP8 transcripts in rat choroid plexus [122]. Immunohistochemical studies performed in chicken, rat or mouse brain demonstrated the choroidal localization of the receptor, with a predominant apical staining and some vesicular cytosolic staining [113, 122, 123]. LRP8 has also been immunodetected in mouse brain microvessels where it seems to be developmentally regulated. The immunoreactivity was observed in brain capillaries in 18-day-old animals, but was absent or below detection limit in adult mouse brain. The choroidal epithelium was immunopositive at both stages. LRP8 shares many ligands with LRP1 and could participate in the clearance of α2-macroglobulin complexes from CSF. It is also a major receptor for selenoprotein P, which carries most of the selenium in plasma and is responsible for delivering this metal to the whole organism [124]. LRP8 has been shown to mediate endocytosis of selenoprotein P in Sertoli cells in the testis [125]. Accordingly, LRP8 knockout animals had decreased levels of selenium in the testis. They also presented lower amounts of selenium in the brain, suggesting that the cerebral entry of this metal is dependent on LRP8. Selenoprotein P immunoreactivity was lost in the choroidal epithelium of LRP8 deficient animals compared to the wild type mice [123]. Collectively, these data indicate that selenoprotein P is taken up in choroidal epithelial cells by LRP8 mediated endocytosis. Whether this leads to transcytosis and release of selenium in CSF as a complex with its carrier, or endosomal dissociation of the complex followed by selenium sorting and recycling of LRP8 to the membrane remains to be deciphered. Given the restricted pattern of expression of LRP8 in the whole body and its substantial level of expression at

the BCSFB, LRP8-mediated transcytosis provides a delivery route with a definite selectivity for the brain. Further studies are awaited to elucidate the transcytosis mechanism and identify potential peptide ligands or other endocytosis triggering effectors.

3.3.2. Transcytosis Pathways Specific to the BCSFB

3.3.2.1. The Folate Pathway

Folates are essential vitamins that serve as cofactors in basic metabolic reactions such as the *de novo* synthesis of purine nucleotides and thymidylate, and in the regeneration of methionine. They are water soluble anions at physiological pH and poorly permeate cell membranes, implying that cellular uptake must be facilitated. Three main systems have been characterized, that have different affinities for the physiologically active 5-methyltetrahydrofolate (5MTHF), and different pH preferences. Two of these systems are facilitative transporters, members of the large solute carrier superfamily [126]. The reduced folate carrier (RFC, SLC19A1) is a ubiquitously expressed low affinity transporter, functioning at physiological pH. It localizes at the apical membrane of the choroidal epithelium. The proton-coupled folate transporter (PCFT, SLC46A1) works at an acidic pH with a low affinity, in the micromolar range. It is largely expressed in the intestinal epithelium where it is responsible for the absorption of dietary folates, and is also present in a number of other tissues including the choroid plexus. PCFT immunolabeling stains the basolateral membrane as well as the cytoplasm of choroidal epithelial cells [127, 128]. The third system involves folate receptors (FR), which mediate endocytosis of folates in a clathrin-independent manner upon binding at neutral pH. These receptors have restricted tissue distribution. FR-alpha (FRα) is found only in a few specialized epithelia, notably the choroidal epithelium which displays the highest level of expression among positive tissues [128]. Intense immunoreactivity in human choroid plexus has been reported, with staining of both the apical and basolateral membranes [127, 128]. FRα has a low binding constant in the nanomolar range, similar to the plasma concentration range.

Physiological levels of folate in CSF are 3- to 4-fold higher than in matched blood samples. Cerebral folate deficiency, which is caused by an invalidating mutation in the gene FOLR1 coding for FRα, results in very low folate levels in CSF, in the absence of any change in blood levels. The choroid plexus, being the only tissue expressing FRα in the brain, must be an essential route of folate delivery to the brain, with a crucial role played by this receptor. The delivery pathway that had emerged postulated that in physiological conditions 1) folates are taken up at the basolateral membrane by FRα-mediated endocytosis, 2) are released from the receptor in acidifying endosomes and exported from those vesicles by PCFT, and 3) finally transported across the apical membrane by RCF to reach the CSF [129].

A recent work reevaluating folate delivery across the choroidal epithelium by a combination of *in vitro* and *in vivo* approaches unraveled a new transport mechanism [127]. Fluorescent folates and FRα were cotransported from the basolateral to the apical membrane of Z310 rat choroidal cells transfected with the human FRα (hFRα), and were released from cells into exosomal vesicles. Consistent with this exosome-mediated delivery, hFRα was identified into intralumenal vesicles within multivesicular bodies in the transfected cells. When injected intraventricularly in mice, hFRα positive exosomes purified from transfected Z310 cells were able to enter the brain parenchyma at some distance from the ventricular wall, and colocalized with distinct neural cell types, in particular astrocytes. By contrast, hFRα negative exosomes remained at the periventricular border. Exosomes containing FRα were also identified in human CSF, where their abundance was correlated with CSF concentration of 5MTHF. Relevant to the potency of such delivery pathway, choroid plexuses contribute to a substantial part of exosomes found into the CSF, as 38 % of them were FRα positive

in control individuals. This new mechanism, coupling receptor-mediated transcytosis of ligands through the choroid plexus with the apparently targeted distribution of the ligand to brain cells via exosomes (path 3 in Fig. **2**), opens a new line of research in the field of cerebral drug delivery.

Importantly, cancer cells frequently overexpress FRα, as a mean to answer to their metabolic needs and to support cell growth and division. Of particular interest is the high expression of FRα in pediatric ependymal tumors [130]. Folate-conjugated anticancer compounds entering the CSF through the choroid plexus would have an immediate access to these tumors and an enhanced penetration in the target cells.

3.3.2.2. Plasma Protein Transport

When plasma protein transfer from blood to CSF was observed in newborn rats some decades ago, using labeled albumin and immunoglobulins, it was accounted for by the immaturity of the BCSFB [131]. This explanation is now largely disregarded, and evidence in favor of a specific and developmentally regulated mechanism of protein transfer across the choroidal epithelium has accumulated. Immunoreactivity of several individual plasma proteins was observed in choroidal epithelial cells during fetal life in various mammal species including human [45, 132, 133]. Actual protein transfer from blood to CSF was demonstrated using exogenous human albumin in sheep fetuses or rat neonates [134, 135]. This pathway of protein uptake and transfer by choroid plexus displays an apparent specificity as all plasma proteins cannot be detected within choroidal epithelial cells [134]. This observation, added to the fact that there is no correlation between CSF/plasma concentration ratios and the molecular radius of the transported proteins, suggested that a receptor-mediated transfer mechanism is involved [133, 136]. Extensive work has been conducted to identify the choroidal receptors for plasma proteins, and to characterize the cellular mechanism supporting transport to the CSF. Several pathways may be expected, as transported proteins are structurally unrelated [137]. When administered to Monodelphis fetuses, bovine fetuin was taken up by choroidal epithelial cells when the exogenous protein was injected intraperitoneally, but not after intraventricular administration [45]. Thus, at least for this protein, the transfer mechanism seems unidirectional, functioning solely from blood to CSF, unless this *in vivo* directionality was determined by the physiological concentration gradient.

Several proteins with an albumin-binding capacity have been identified in a developmental transcriptomic study of the mouse choroid plexus [138]. Both glycophorin A and C, as well as SPARC (secreted protein acidic and rich in cysteine) were expressed at high levels at the embryonic stage. Analysis of their expression at the single cell level indicated a substantial degree of colocalization with the plasma protein transfer capacity. Given the distinct subcellular distribution of glycophorin A and SPARC in the choroidal tissue, it was proposed that the different albumin-binding proteins function sequentially in the transcellular transfer of albumin from blood to CSF. *In situ* proximity ligation assay performed on mouse endogenous albumin and SPARC indicated that both proteins are close enough to interact, and reinforced the proposed function of SPARC in the process. Yet, the fact that not all cells immunoreactive for albumin express SPARC [138] infers the existence of other receptors/transporters, which remain to be identified. The signal generated in the proximity ligation assay by SPARC/albumin marked a branching continuous transcellular tubular system that needs to be characterized (path 4 in Fig. **2**). The exact mechanism of protein release in the ventricular CSF also requires further investigation. Independent observations suggest that it might involve exosome formation. Immunolocalization of SPARC in apical vesicles of mouse choroid plexus epithelial cells and immunolabeling of albumin in multivesicular bodies in sheep choroid plexuses are both consistent with that hypothesis [138]. It would also be consistent with the presence of albumin/SPARC complexes (detected by the in

situ proximity ligation assay) in the ependymal and subependymal layer [139].

The route of plasma protein transport through the choroid plexus appears crucial for normal brain development. It generates a high protein concentration in CSF during fetal life, which increases intraventricular osmotic pressure, promotes the transfer of water necessary for ventricular expansion and brain morphogenesis [140]. Plasma proteins may also deliver to the developing brain the various morphogens, hormones and growth factors that they carry. The number of plasma protein immunoreactive cells increases continuously with age, although their percentage relative to the total number of choroidal cells tend to decrease because of the rapid and important choroid plexus growth. A developmental analysis in Monodelphis indicated that plasma protein transporting cells represent about 10% of the total epithelial cell population up to 9 days after birth (equivalent to newborn rat), and their relative abundance slowly drops to 4-5 % at the adult stage [45]. Therefore, the absolute protein transfer capacity of the BCSFB may remain significantly relevant to drug delivery, despite the concurrent drop in CSF protein concentration, which results from changes in CSF dynamics [37]. The molecular mechanisms governing cellular uptake of plasma proteins, vesicular trafficking and release in the CSF in selected cells of the choroidal epithelium need to be deciphered precisely. Manipulating this pathway may prove a strategy of interest for macromolecule delivery to the developing and possibly the adult brain.

4. PERSPECTIVES

Besides the long characterized receptors described above, novel promising transcytosis pathways at the BBB have emerged. One of them was uncovered by through the identification of FC5, a llama single-domain antibody that was selected for its ability to be transported across an in vitro model of the BBB [141]. The endothelial glycosylated receptor mediating FC5 endocytosis at the luminal membrane still awaits precise identification, but FC5 has proved to be an efficient shuttle to deliver a therapeutic antibody across the BBB [15]. Transport of FC5 or FC5-derived bispecific antibodies across the BCSFB has not been addressed. The puzzling pattern of distribution of fluorescently labeled FC5 obtained by ex vivo brain optical imaging following systemic administration, which suggests a ventricular accumulation of the compound [73], calls for further examination of this possibility. Other promising targets were recently identified by searching transmembrane proteins highly expressed in mouse brain endothelium by a proteomic approach [142]. One candidate is CD98hc/SLC3A2, which forms the heavy chain of the amino acid transporter LAT1. Antibodies against CD98hc showed a strong uptake in brain parenchyma. When used as bispecific antibodies with a BACE1 binding specificity in the therapeutic arm, antiCD98hc was able to deliver active anti-BACE1 in sufficient amounts to observe a reduction in brain Aβ level. As previously mentioned LAT1 is not exclusive to the BBB and is also present in the basolateral membrane of the choroidal epithelium. It can be speculated that LAT1-mediated transport of bispecific CD98hc/BACE1 antibodies occurred at both barriers, and that CSF entering antibodies contributed to lower the cerebral Aβ load as discussed for the bispecific TfR1/BACE1 antibody.

Screening of peptide and antibody libraries for BCSFB ligands is still to be performed. It should lead to the discovery of novel ligand/receptors systems, possibly specific for this barrier, in addition to the folate receptor. A pilot study has been conducted [143], which enabled the selection of three peptides with a capacity to bind to, and internalize in choroidal epithelial cells. One limitation of the study, in terms of drug delivery, relates to the fact that screening of the phage display peptide library was performed on isolated choroid plexuses. The selected peptides of interest were validated for binding and cellular uptake from the apical membrane only, either in vitro in a choroidal epithelial cell line, or in vivo

following intracerebroventricular injection. The eventuality that any of these peptides targets a bidirectional transcytosis mechanism and is also endocytosed at the basolateral membrane and released in CSF has not been tested. Peptide and antibody libraries need to be screened again to identify ligands for potential basolateral membrane proteins.

Databases generated from transcriptomic and proteomic studies on choroid plexus, microdissected choroidal epithelial cells, and CSF exosomes are available and can be thoroughly probed to select receptor candidates highly expressed at the BCSFB. Developmental regulation should be taken into account, as stage-specific properties of this barrier could be exploited to specifically address perinatal and pediatric diseases.

Mechanisms supporting transcytosis pathways must be fully dissected in order to understand at the molecular level, the subsequent events that control vesicular trafficking and the respective sorting of the receptor and its cargo within the endosomal network. Factors that govern the fate of endocytosed cargoes towards recycling endosomes, late endosomes/multivesicular bodies, or lysosomes, such as the ligand affinity for its receptor, should be identified. Similarly, how cargo emptying occurs or exosomes form and disseminate in the peculiar environment of the thick microvilli associated with the apical choroidal cell membrane needs to be deciphered. CSF secretion may influence these processes. These elements are crucial determinants of the overall capacity of the transcytosis process and may be manipulated for optimizing drug delivery.

Polarized epithelial cells have provided clues on all these aspects, but a given receptor may engage in different pathways among epithelia. For instance, LRP2 localized at the apical membrane of renal proximal tubule cells mediates endocytosis of leptin and trafficking to lysosomes for degradation [144], while LRP2 in the choroidal epithelium mediates basolateral-to-apical transcytosis of leptin and insulin-like growth factor I, and possibly reverse transcytosis of Aβ in the opposite direction (see above). The membrane domain targeted by newly synthesized LRP2 in the choroidal epithelium is currently unknown. The mechanisms sustaining the switch between endocytosis/catabolism and transcytosis, and the factors governing transcytosis directionality are still elusive in this epithelium. They need to be understood to maximize receptor-mediated transport of therapeutic cargoes across the BCSFB.

Exosomes generated from the choroidal epithelium represent more than a third of CSF-borne exosomes. They appear as promising nanocarriers for the delivery of therapeutic molecules. Exosomes are acknowledged as important for volume transmission within the brain [145] and have been reported to recognize specific cellular populations. Yet, many questions remain to be answered to understand the biology of CSF-borne exosomes. There is a need to fully characterize choroid plexus-derived exosomes, their biogenesis and mechanisms of secretion as discussed above, and to identify the specific neural cell populations that they can target. This will require a detailed identification of the proteins localized on the exosomal membrane that serve as receptors and mediate the recognition of the correct target cell, and a clear understanding of the mechanisms that drive exosome penetration into cerebral structures at variable distances from the CSF.

While changes in BBB transport properties are increasingly recognized in various pathologies, the choroid plexus is seldom studied in the context of pathophysiological events. There is however evidence that choroidal dysfunctions are associated with brain diseases. In Alzheimer's disease the morphological and functional alterations of the choroidal tissue are striking (reviewed in [24]). LRP2 for instance is downregulated in the choroid plexuses in a mouse model of this disease [121]. The number of exosomes in CSF, including those of choroidal origin, is decreasing with age and their composition changes [146]. More generally, transport mecha-

nisms are dynamic processes often impacted by oxidative stress and inflammation, which is a common feature of many central disorders. These different set of data call for a thorough exploration of transcytosis mechanisms at the choroid plexus in the context of the brain pathologies for which drug delivery across the BCSFB would clearly be beneficial.

Finally, and possibly more prospectively, cellular therapy may benefit from targeting the blood-CSF barrier in two ways. This barrier has recently emerged as a long underestimated route of entry of immune cells in the brain for immune surveillance, at early stages of inflammatory events, and in diverse infectious diseases. As stated in the first part of this review, in these inflammatory/infectious contexts, the CSF-filled spaces are obvious targets for therapeutic intervention. Engineered immune cells or stem cells that could home to CSF through the choroid plexus can be envisioned as vehicles to deliver therapeutic agents, or as therapeutic cells counteracting deleterious immune cell actions. This therapeutic approach could also be relevant to the treatment of brain tumors. The second domain of interest in the context of cellular therapy is related to the capacity of choroidal epithelial cells to secrete a relatively large number of morphogens, growth factors, guidance molecules and hormone carriers. All these factors are critical for the regulation of neural stem cells during developmental neurogenesis and in adult, for the overall development and maturation of the brain during pre-and postnatal development, and for brain homeostasis throughout life. Considering the role of CSF in volume transmission, it is tempting to imagine engineered epithelial choroid plexus cells that, incorporated among the native cells, would be a source of repair factors, or compensate choroidal epithelium alterations in both perinatal injuries and adult degenerative diseases. Proof of concept that this approach can be envisioned has been provided by the demonstration that choroidal cells can be differentiated from stem cells [147]. This adds a new and original facet to the cell therapy-based neuroprotection and repair strategies currently designed to treat CNS diseases.

In conclusion, an increasing body of evidence points to the choroid plexus-CSF system as a seldom explored but promising pathway to deliver therapeutics to the brain. This field of research is in its initial stage of investigation, but should benefit from the numerous works exploring drug delivery across the BBB.

CONFLICT OF INTEREST

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