Drug Targeting to the Brain

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brain drug targeting, local delivery, global delivery, viral delivery, nonviral delivery

Abstract

The central nervous system (CNS) is a sanctuary site and is protected by various barriers. These regulate brain homeostasis and the transport of endogenous and exogenous compounds by controlling their selective and specific uptake, efflux, and metabolism in the brain. Unfortunately, potential drugs for the treatment of most brain diseases are therefore often not able to cross these barriers. As a result, various drug delivery and targeting strategies are currently being developed to enhance the transport and distribution of drugs into the brain. Here we discuss briefly the biology and physiology of the blood-brain barrier (BBB) and the blood-cerebro-spinal-fluid barrier (BCSFB), and, in more detail, the possibilities for delivering large-molecular-weight drugs by local and global delivery and by viral and receptor-mediated nonviral drug delivery to the (human) brain.

BARRIERS IN THE BRAIN

BBB: blood-brain barrier

BCSFB: blood-cerebrospinal-fluid barrier

CSF: cerebral spinal fluid

LRP: low-density lipoprotein receptor-related protein

There are three barriers that limit drug transport to the brain parenchyma. These are the blood-brain barrier (BBB), localized in the capillaries in the brain; the blood-cerebrospinal-fluid barrier (BCSFB), which is presented by the choroid plexis epithelium in the ventricles; and the ependyma, which is an epithelial layer of cells covering the brain tissue in the ventricles and limits the transport of compounds from the cerebral spinal fluid (CSF) to the brain tissue.

Blood-Brain Barrier

Ehrlich (1885) was the first to show evidence for the existence of a barrier between blood and brain. He injected vital dyes intravenously and found that, in contrast to other tissues, it did not stain the brain (1). His successor Goldman (1913) injected these dyes into the CSF, after which staining of the brain was observed, but not of the peripheral organs (2). Since this seminal discovery, much research has been performed on the (patho) physiology and pharmacology of the BBB (see also other reviews in References 3–9).

The current knowledge is that the BBB is situated at the interface of blood and brain and its primary function is to maintain the homeostasis of the brain. Furthermore, the BBB is not uniform throughout the brain because the capillaries in the circumventricular organs (CVOs) are fenestrated (10). **Figure 1** gives a schematic representation of the barriers present in the central nervous system (CNS).

The human BBB has a total blood vessel length of approximately 600 km. In fact, every cubic centimeter of cortex contains the amazing sum of 1 km of blood vessels. It has an estimated surface area of approximately 20 m^2, which is similar to the BCSFB (11). However, the surface area of the BCSFB faces the CSF and not the blood, which makes the BBB, based on total blood flow and its wide vascular bed, functionally the most important global influx barrier preventing solutes from entering the brain (10).

The BBB is mainly formed by brain capillary endothelial cells (BCEC) (12), although other cell types, such as pericytes, astrocytes, and neuronal cells, also play an important role in the function of the BBB (13–15). BCEC are different from peripheral endothelial cells, as can be seen schematically in **Figure 2** (in which the specific surroundings of the brain capillaries are shown). BCEC have specific characteristics, such as tight junctions, which prevent paracellular transport of small and large (water-soluble) compounds from blood to the brain (12, 16, 17). Furthermore, transcellular transport from blood to brain is limited as a result of low vesicular transport, high metabolic activity (18), and a lack of fenestrae (8, 12). It functions as a physical, a metabolic (18, 19), and an immunological barrier (20). In addition, receptors at the BBB may play a role in brain signaling [e.g., insulin receptor (21), low-density lipoprotein receptor-related protein (LRP)1,2-receptor (22)]. These specific characteristics of the BBB are induced and maintained by the (endfeet of) astrocytes surrounding the BCEC (12–14), as well as by neuronal endings, which can directly innervate the BCEC (12, 23). Pericytes also play a role at the BBB, as they share the continuous capillary basement membrane with the BCEC (15). Their phagocytotic activity forms an additional BBB property (10). Furthermore, pericytes have

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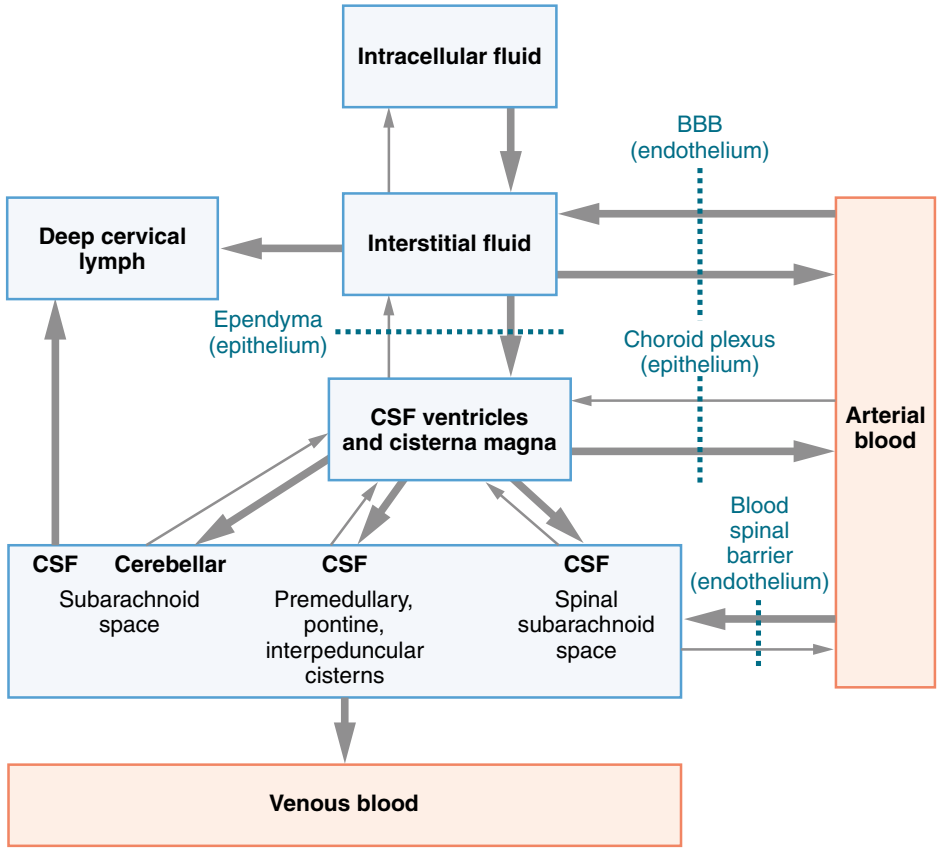

Figure 1

The various barriers that one can find in the brain (*broken lines*), representing the blood-brain barrier (BBB), the blood-cerebrospinal fluid (CSF)-barrier, the brain-CSF-barrier, and the blood-spinal barrier. The BBB has the largest surface area, and is, therefore, considered to be the most important influx barrier for solutes to enter the brain. Also shown are the paths of fluid movement (*solid arrows*) between cerebral intracellular fluid (ICF), interstitial fluid (ISF), CSF, blood, and lymphatics. Thick arrows represent major paths of fluid movement under normal conditions. Thin arrows represent minor paths of fluid movement under normal conditions. Redrawn and modified from Reference 52.

been shown to regulate endothelial homeostasis, which then induces the endothelial release of plasminogen-activator inhibitor-1 (PAI-1), thereby negatively regulating brain endothelial fibrinolysis (24).

At present, many transport systems have been discovered that play an important role in maintaining BBB integrity and brain homeostasis and also influence drug transport to the brain. These comprise carrier- and receptor-mediated transport systems (CMT, RMT), including cationic and anionic influx and efflux systems such as P-glycoprotein (25, 26), multidrug-resistance (MDR) proteins (27, 28), nucleoside transporters, organic anion transporters, organic cation transporters, large amino

CMT: carrier-mediated transport system

RMT: receptor-mediated transport system

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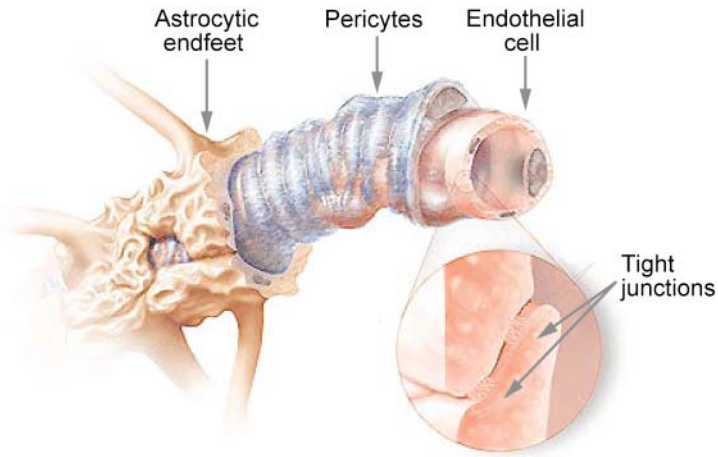

Figure 2

Schematic representation of surrounding pericytes (covering about 20%–30% of the capillary surface) and astrocytic endfeet projecting on the endothelial cells of the cerebral capillaries that induce and maintain the blood-brain barrier. In contrast, endothelial cells of peripheral capillaries do not form a tight barrier because they lack the specific input of these brain cells. See text for details. Reprinted with permission from G. Miller, *Science* 297:1116–18. Illustration by C. Slayden. © 2002 AAAS.

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acid transporter, and the RMT systems such as the transferrin-1 and -2 receptors, the melanotransferrin receptor, and the scavenger receptors SB-AI and SB-BI (28–30). Moreover, these transporters play an important role in the drug disposition process, with particular importance in terms of the clinical implications of transporters for drug-drug interactions, drug toxicity, interindividual variability in drug response, and disease (31).

Because of the complex interactions between cell types and the transport of compounds to and from the brain, as well as the dynamic regulation of BBB properties (e.g., receptor expression, formation of tight junctions), the "multitasking" BBB (32), or the so-called neurovascular unit (5), is considered to be an organ protecting and regulating the homeostasis of the brain (28). In addition, these properties are influenced by disease and drug effects that will change the functionality of the BBB under such conditions and influence drug delivery to the brain (6, 7, 33). Moreover, the disposition of drugs in the brain (neuropharmacokinetics) in healthy and disease conditions should be studied to enhance the treatment of diseases involving the brain (34).

The Blood-Cerebro-Spinal-Fluid Barrier and Ependyma

The BCSFB is a rather complicated system (for other reviews see also References 35–37). It comprises mainly the choroidal and arachnoidal epithelium, giving access to the ventricular and subarachnoidal CSF, respectively. Considering its location and the direction of the CSF flow, the choroidal epithelium at the choroid plexi (CP) is

considered the most important part of the BCSFB located in the lateral ventricles and in the third and fourth ventricles. The CP functions as a physical, enzymatic, and immunological barrier, and it plays a role in drug metabolism, drug transport, repair, and signaling. It contains phase I–III enzymes (36). Phase I enzymes are responsible for functionalization of drugs and include cytochrome P-450 isoform (CYP2B1,2) and monoamine oxidase. Phase II enzymes are responsible for conjugation of drugs and include glutathione and glucuronosyl transferases. Phase III enzymes include transporters such as Na-dicarboxylate cotransporter, ascorbic acid transporter, organic anion transporter (OAT), organic anion transporter polypeptide 1 and 2 (Oatp1,2), organic cation transporter (OCT), equilibrating and concentrative nucleotide transporters (ENT and CNT), and multidrug transporters such as Pgp and MRP1 (4, 26–28). Furthermore, the CP seems to be involved in repair of neurons by secreting neuroprotective compounds and acting as a site of neurogenesis. In addition, the CP expresses many receptors, and several of them are involved in signaling between the immune system and the brain; pathology of the CP has been found in many CNS disease conditions (36).

In humans, the total volume of the CSF is approximately 160 ml and the formation rate is approximately 0.35–0.40 ml/min. It is produced mainly by the CP (60%) and the remainder (40%) via ultrafiltration by brain capillaries (38). It flows from the lateral to the third and fourth ventrical and subsequently into the cisterna magna and other large basal cisterns (see **Figure 1**). From there the CSF flows posteriorly and downward into the subarachnoid space around the spinal cord and upward around the cerebral hemispheres. The CSF flows into venous blood via the arachnoidal villi in the subarachnoidal space.

Because the endothelium in the blood capillaries in the CP is fenestrated, resistance to drug transport seems to be produced by (gap)-junctions of the CP-epithelium, which are more permeable than the tight junctions of the BBB-endothelium (35). Moreover, blood flow in the CP-blood capillaries seems to be 5 to 10 times higher than the mean cerebral blood flow (36). In addition, it has been calculated that the total surface area of the entire CP is in the same order of magnitude as the entire BBB (11). Taken together, this raises the possibility for substantial drug transport via the CP into the CSF. However, the surface area of the BCSFB faces the CSF and not the blood, which makes drug transport to the CSF less effective. Moreover, from the point of view of drug transport to the brain parenchyma, the BCSFB has some disadvantages. Once a drug is in the CSF, an additional barrier for the transport of molecules is presented by the ependyma covering the ventricles. The ependyma is a single layer of epithelial cells connected by (gap)-junctions and seems to be rather permeable, particularly to small molecular lipophilic compounds, and permeability for macromolecular drugs also has been claimed (see also Direct Injection/Infusion of Macromolecular Drugs, below) (35).

STRATEGIES FOR DRUG DELIVERY TO THE BRAIN

For many diseases of the brain, such as Alzheimer's disease, Parkinson's disease, stroke, depression, schizophrenia, epilepsy, and migraine headache, the drugs on the market

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Global delivery: administration of drugs via the vascular system

are far from being ideal, let alone curative, drugs. A significant part of the problem is the poor BBB penetration of the drugs against neuronal targets for treatment of these disorders. This includes approximately 98% of the small molecules and nearly 100% of large molecules, such as recombinant proteins or gene-based medicines. Therefore, much effort is going towards delivery and targeting of drugs to the brain (10, 39). Drug delivery to the brain can be achieved via several methods, including local invasive (direct injection/infusion) delivery, induction of enhanced permeability, and the application of global physiological targeting strategies. Apart from the delivery of small molecules (e.g., <500 Da) to the brain, the global delivery of large hydrophilic molecules, including enzymes, interference RNA (RNAi), and genes, presents a serious problem. In addition, once such drugs have been delivered to the BBB, following internalization they may be targeted for degradation by the endosomal/lysosomal or ubiquitin/proteasomal system (18). Therefore, systems should be applied (e.g., endosomal escape mechanisms) to bypass these routes of degradation.

LOCAL BRAIN DELIVERY BY DIRECT INJECTION/INFUSION OF DRUGS INTO THE BRAIN

Because of the lack of success in targeting the brain via the vascular route, invasive methods, such as direct injection or infusion and convection-enhanced delivery, have been applied to macromolecular drugs as well as to the delivery of viral vectors. This is reviewed in the following paragraphs.

Direct Injection/Infusion of Macromolecular Drugs

Intra-cerebral-ventricular (ICV) or intrathecal drug infusion comprises direct injection/infusion of drug into the CSF. However, as mentioned before, CSF is completely drained into the venous circulation, and drugs still have to cross the ependymal brain-CSF barrier. This has been shown as feasible particularly for many small [mostly lipophilic (39)] drugs following intraventricular administration. In addition, it has also been shown that compounds with a molecular weight less than 5000 rapidly penetrate the ependyma, but that penetration into the brain parenchyma was limited owing to diffusion, tortuosity, transcapillary loss, cell uptake, and binding (40). As a result, drug infused into the CSF has minimal access to the parenchyma by diffusion (**Figure 3*c***) (41). In addition, compounds with a molecular weight greater than 5000 penetrate the brain parenchyma very poorly, even when administered intraventricularly. Following a 3–5 h infusion of ^{14}C-inulin in the lateral ventricle of dogs, only in tissues lining the ventricle was an appreciable uptake found (42) and intraventricular injection of ^{125}I-brain-derived neurotrophic growth factor (43) in rats resulted in very poor uptake by the brain parenchyma. Very recently, it was elegantly demonstrated that the uptake of ^{125}I-insulin-like growth factor-1 (mol. weight 7.7 kDa) following intraventricular infusion was very poor by brain parenchyma and was rapidly cleared from the CSF compartment mainly into blood (44). In addition, these authors stated that, under healthy conditions, the direction of CSF flow will probably differ from that under disease conditions and that further investigations are required in this

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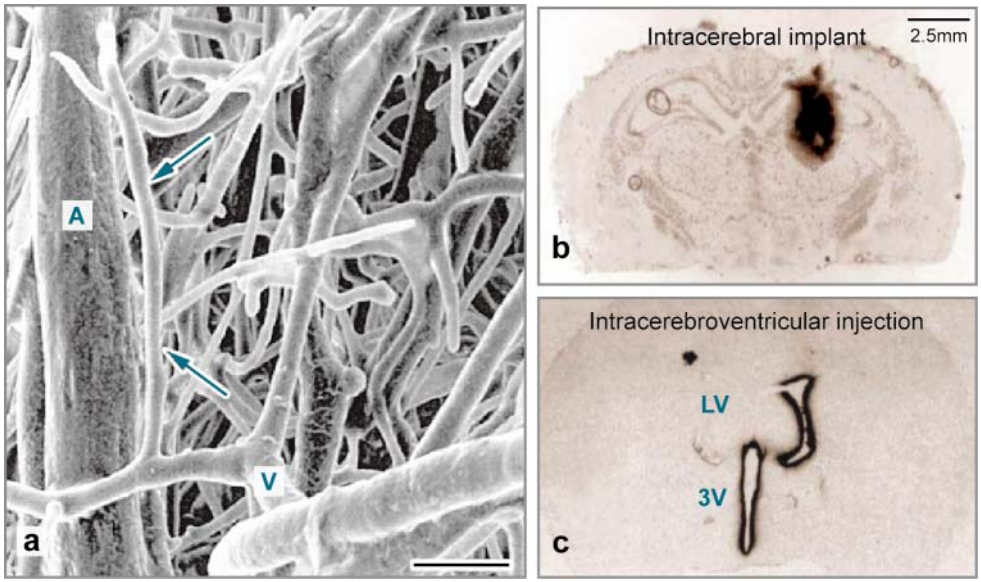

Figure 3

Drug delivery via the vascular route will enable widespread distribution of the drug to each single neuron within the brain (note that the bar in panel A indicates a length of 25 μm, which is about the size of a single neuron). (*a*) Scanning electron micrograph of a vascular cast of a mouse brain (A, artery, V, vein). Reprinted from Reference 176, with permission from Lippincott Williams & Wilkins. (*b*) Minimal diffusion of [^{125}I]-nerve-growth factor (NGF) after intracerebral implantation of a biodegradable polymer (note that the bar indicates a length of 2.5 mm, which was also the size of the implant). Reprinted from Reference 177, with permission from Elsevier. (*c*) ICV injection of [^{125}I]-brain-derived neurotrophic factor (BDNF). Reprinted from Reference 178, with permission from Elsevier. Note that the neurotrophin does not distribute into the brain beyond the ipsilateral ependymal surface.

respect. Furthermore, experimental data in patients with Parkinson's disease indicate that intraventricular GDNF-infusion by a Medtronic device was biologically active but did not improve Parkinsonism, possibly because GDNF (a dimer with a mol. weight 33–45 kDa) did not reach the target tissues in sufficient concentrations (45).

Direct infusion into the brain has also been tried. The delivery of GDNF by direct infusion into the putamen of Parkinsonian patients in one study showed general improvement over time (46), whereas a phase II clinical trial only demonstrated potential efficacy (47). Indeed, the pharmaceutical company Amgen confirmed its earlier decision to stop a phase II clinical trial with GDNF infusions into the putamen of 48 patients stating: "scientific results indicated that allowing patients to continue treatment could potentially cause permanent harm, complicating an already devastating disease" (48).

A variant to direct infusion is convection-enhanced drug delivery, where a positive pressure infusion in brain parenchyma has been applied to increase drug uptake. Uptake was significantly improved when the viscosity of the infusate was increased

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(49), perhaps by a similar mechanism (osmotic opening) as has been used by Kroll & Neuwelt (50). Good results were seen with a large-volume infusion of naked DNA in the tail vein or in arms and legs when blood flow was temporarily inhibited; however, this is not applicable to the brain (51).

Brain implants have also been used for the local delivery of drugs to the brain (**Figure 3*b***). Indeed, it has been shown that polymeric implants [poly (ethylene-covinyl acetate)] containing ^{125}I-nerve–growth factor in rat brain were effective for local delivery but unfortunately not for global delivery of the drug (52).

In conclusion, because of the fenestrated capillaries in the CPs and their total surface area that seems to be comparable to that of the BBB, drug transport into the CSF could be substantial. However, in addition to the need for direct injection into brain parenchyma or infusion into the ventricles, for drugs that must reach their target by passive diffusion, the direction of the CSF flow in the ventricles, the presence of the ependymal barrier (particularly for compounds with a mol. weight >5000), and the direction of the flow of the interstitial fluid in the brain parenchyma are major obstacles to effective global penetration of such drugs into the brain.

Enhanced drug delivery: increasing the access of drug to the target site

Local delivery: direct administration of drugs at the target site

AAV: adeno-associated viral vector

LV: lentiviral vector

Direct Injection/Infusion of Viral Vectors

Gene therapy (53–56) is meant to deliver genetic material with a therapeutic goal of encoding proteins (e.g., enzymes) or siRNA/shRNA to somatic cells (57). In addition, gene therapy provides the potential for a long-term effect following one single administration. However, genes are hydrophilic, charged, and large molecules that cannot pass cell membranes and tight cellular layers like the BBB. Therefore, means have to be found and developed to transport genes to the desired site of action.

Viruses have evolved over millions of years to obtain optimal mechanisms for gene delivery to host cells, which makes them applicable as a biological vector system to deliver genetic material to brain cells. There is a broad range of viral vectors available, but the most commonly used are adeno-associated viral vectors (AAV) and lentiviral vectors (LV). Important issues in viral gene delivery are stable transgene expression, limited immunogenicity, induction of an inflammatory response, the (lack of) cell-specific targeting efficiency, safety, toxicity, and the need for packaging cell lines (58).

Brain tropism (e.g., brain targeting) varies from virus to virus and has been shown to be insufficient to pass through the BBB following intravenous administration, therefore the virus has to be injected directly into the brain. Moreover, various viral vectors have been shown to have different affinities for various brain cells (53–59). This could be improved when viral vectors can be produced with a homing/targeting device at their surface. Furthermore, the selectivity of gene transcription in the brain can be strongly influenced by application of brain selective promoters such as the myelin basic protein (MBP) promoter, the neuron-specific enolase (NSE) promoter, the platelet-derived growth factor-beta (PDGB-beta) promoter, the glial fibrillary acid protein (GFAP) promoter, and synthetic promoters such as the CMV plus human growth hormone first intron enhancer and the CMV-chicken beta-actin promoter (53–59). Moreover, the addition of a posttranscriptional regulatory element of the

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wood-chuck hepatitis virus (WPRE) has been shown to enhance the expression of GFP (60). Similarly, the application of the replication origin (oriP) and Epstein-Barr nuclear antigen (EBNA-1) elements have been used to enhance episomal transcription of plasmids. However, application of such elements could induce tumorgeneity, which therefore makes them not readily suitable in humans (61).

The Tet and Cre-Lox systems are other regulatory systems that have been tried, particularly for viruses that integrate their genetic material into the genome. The Tet system (tetracycline transactivator under the control of the tetracycline inducible promoter) has shown to upregulate the expression of GFP in rat brain in the absence of doxycycline (62), whereas the Cre-Lox system allows a conditional but irreversible gene modification (63).

The adeno-associated virus (AAV) is a human parvovirus that contains a ssDNA. There are approximately 35 known serotypes. Several of them have been engineered into recombinant viral vectors from which the rAAV1, rAAV2, and rAAV5 are most widely used to deliver genes to the brain (56, 64, 65). These serotypes are particularly effective in the transduction of neurons (65). The attractive feature of the virus is that its replication is dependent on coinfection with a lytic helper virus, which precludes the reversion into a replication competent virus.

rAAV-vectors are capsid virions composed of a nucleic acid genome surrounded by a proteinaceous shell. The composition of the capsid determines which cells they bind to and enter. They enter the cell following binding to heparin sulfate proteoglycan (HSPG) receptors. Two other coreceptors seem to help the process, and finally the dsDNA enters the nucleus through the pore (66, 67).

Once in the cell, the rAAV genome is maintained as an extrachromosomal or episomal element (53, 68, 69) that eliminates the risk of proto-oncogene activation or endogenous gene inactivation, which may occur following insertion into the host genome (69). Nevertheless, random integration of rAAV material does irregularly occur (53, 69). The episome might replicate along with the endogenous cellular DNA, but it can also be maintained as a nonreplicating extrachromosomal element (53). In nondividing cells, the episome can be maintained as a stable element, resulting in long-term transgene expression in cells (53, 68–70). In addition, a safe and long-term correction of genetic diseases following a single administration has been demonstrated in animal models (54). These viruses can be produced under current Good Manufacturing Practices (cGMPs) (54) guaranteeing that pure and high-quality rAAV vectors can be produced for human use.

In animals expression of rAAV2-delivered vectors has been shown for brain-derived-neurotrophic factor (BDNF) in atrophy of spinal neurons (71); glial cell line–mediated neurotrophic factor (GDNF), tyrosine hydroxylase (TH), aromatic-L-amino acid decarboxylase (AADC), and guanosine triphosphate cyclohydrolase (GCH) in Parkinson's disease (72–72). In addition, rAAV vectors have been shown to be effective in the treatment of lysosomal storage diseases such as mucopolysaccharoidosis type I, II, and VII (75) and Niemann-Pick A (76). Furthermore, it was demonstrated in mice that by targeting the deep cerebellar nuclei with AAV1 vectors capable of axonal transport, it was possible to get a widespread distribution throughout the cerebellum (76).

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Viral delivery: delivery of plasmids by viral-mediated cellular uptake

The major problem in delivering rAAV vectors to the brain is getting global brain transduction. This holds particularly true for disorders caused by single-gene mutations, such as lysosomal storage disorders and leukodystrophies, where global brain transduction is desirable. Some vectors have been shown to have an enhanced neurotropism when expressing peptides that mimic binding domains for cytoplasmic dynein or NMDA receptors (77). In addition, other viral vectors have been constructed such as chimeric BDNF or GDNF that are expressed as their capsid proteins, which function as homing devices for targeted delivery of genes (78).

Another disadvantage is the occurrence of immune responses when animals have been treated with rAAV vectors, as this has resulted in killing of the transduced cells by cytotoxic T cells (53, 69, 79). It has been suggested that this is caused by a slow uncoating of the rAAV virus in contrast to rapid uncoating (80, 81). In addition, a humoral response can be elicited that results in the production of antibodies. Moreover, memory cells are produced that will boost a strong humoral response when the same antigen is detected again. In this way memory cells prevent the possibility of vector readministration (69).

Another feature is the packaging capacity of viruses. For rAAVs this is rather small (~4.5 kb) (53, 70, 82). However, this may be increased up to 36 kb (82) by using (gutless) vectors where as many viral genes as possible are deleted and that only contain those for the packaging sequence and sequences that define the beginning and end of the viral genome (53, 70).

LV are very promising vehicles to deliver therapeutic genes into the brain (53, 56, 57). LV are capsid virions surrounded by a lipid bilayer envelope. LV enter the cell following binding to HSPG, and following membrane fusion the virus moves along the microtubule by dynein-mediated transport. Finally, the dsDNA enters the nucleus through the pore (53). LV have a moderate packaging capacity, with a maximum of 8 kb. LV can be used to transduce a broad range of dividing as well as nondividing cells. In contrast to the rAAV vectors, LV integrate into the host genome (59) and should therefore provide a longer and more stable transgene expression. However, it could also activate a proto-oncogene (53, 69, 70), but because most CNS cells are terminally differentiated, the risk of tumorigenesis seems to be diminished (53). Nevertheless, the risk that vital genes are inactivated by random integration exists (53, 69).

LV vectors seem to have a low immunogenicity. This seems to be due to the fact that the genomes of these vectors do not encode any viral proteins. Indeed, low immunogenicity and prolonged transgene expression in the presence of preexisting lentiviral immunity were found, which is encouraging for future use of these vectors in brain gene therapy (83).

Although viral delivery systems enter the cell by receptor-mediated endocytosis, this cell entry is not selective/specific because they can enter various cells, including brain cells, owing to the lack of the expression of a selective/specific homing device. Therefore, rAAV and LV can only effectively be delivered to the brain by direct local injection/infusion. However, the diffusion from the site of administration in the brain is minimal, therefore the region of cells that is transduced is restricted to a few millimeters from this site (53, 70). To compensate for poor brain diffusion, usually

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the vector is administered by injections at multiple sites to increase the area of vector delivery. However, injection is also associated with injury at the injection site and as a result could evoke a damaging (inflammatory) response.

LV vectors have been tried for gene therapy of various (brain) diseases such as lysosomal storage diseases, including Sandhoff disease and mucopolysaccharoidosis I (84, 85), Parkinson's disease (86), ALS (87), epilepsy (88), prion disease (89), brain tumors (90), and the delivery of growth factors (91).

Nonviral delivery: delivery of drugs/plasmids by nonviral (receptor)-mediated cellular uptake

Currently, viral gene delivery to the brain can be considered efficient. Many applications of brain gene delivery by rAAV and LV vectors have been demonstrated, and there is an increasing interest in the use of LV vectors. Critical issues at this time are global versus local delivery, including the ability to reach the desired site of action (targeting efficiency). This may be improved considerably when homing/targeting devices are expressed at the outer surface of the virus particle (78), which will allow global delivery of these systems to the CNS following vascular administration, thereby avoiding damage caused by direct injection/infusion. The selectivity of delivery can be further enhanced when tissue-selective/specific promoters are included into the vectors. rAAV vectors stay mainly episomal, whereas LV vectors integrate into the genome with the possibility to activate proto-oncogenes. Immunological reactions may occur but these seem to be reduced following the use of LVs. rAAV and LV have a rather small to moderate packaging capacity and can be safely produced under well-controlled conditions.

With respect to invasive brain drug delivery strategies, one may conclude that these can be effective for local delivery (e.g., tumors), but not for the administration of therapeutic agents directed against more widespread diseases, including diffuse tumors and metastasis, Alzheimer's disease, multiple sclerosis, lysosomal storage diseases, epilepsy, etc. Therefore, because every neuron has its own capillary, the only route for an effective global delivery of biopharmaceutical drugs to the brain is the vascular route. This is further discussed in the next section.

GLOBAL BRAIN DELIVERY BY VASCULAR NONVIRAL DRUG ADMINISTRATION

The advantage of the vascular route is the widespread transport of the infused drug across the whole brain by blood. Each neuron has its own brain capillary for oxygen supply as well as the supply of other nutrients (see also **Figure 3*a***). This means that the vascular route is a very promising one for drug delivery and targeting to the whole brain. However, the main problem for drug transport to the brain parenchyma is BBB passage.

Drug Transport Across the BBB

There are various possibilities for drug transport across the BBB, as shown in **Figure 4**. Passive diffusion depends on lipophilicity and molecular weight. Furthermore, the ability of a compound to form hydrogen bonds will limit its diffusion through the BBB (92). In general, Lipinski's rule-of-five, as well as the Abraham's

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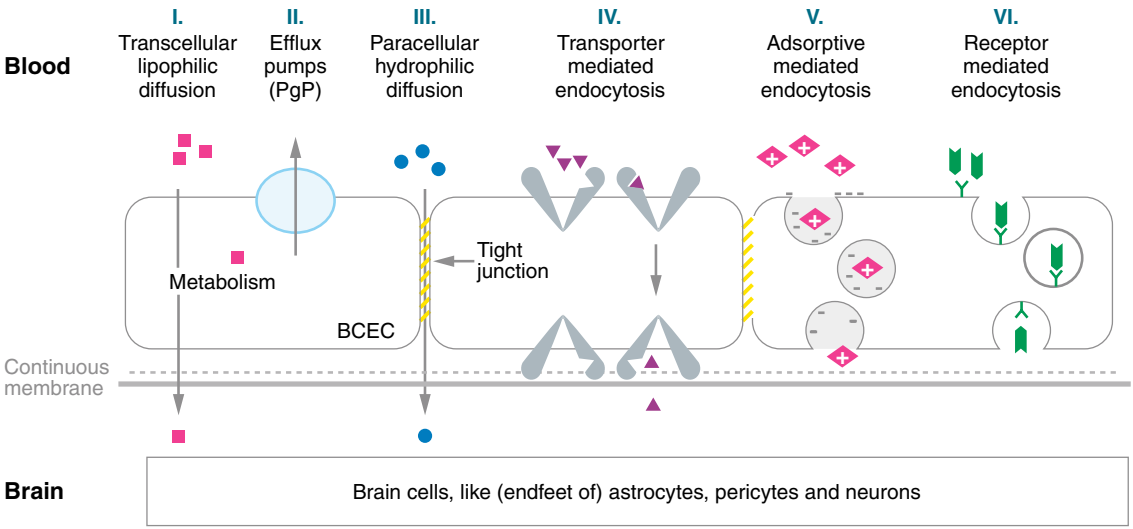

Figure 4

The various transport processes that may occur at the blood-brain barrier (modified from Reference 147).

equation, can be used to predict the passive transport of a drug molecule across the BBB (93, 94). Transport of hydrophilic compounds via the paracellular route is limited, whereas lipophilic drugs smaller than 400–600 Da may freely enter the brain via the transcellular route.

Active transport systems can be divided into absorptive-, carrier-, or receptor-mediated transcytosis. Absorptive-mediated transcytosis is initiated by the binding of polycationic substances (such as most cell-penetrating peptides) to negative charges on the plasma membrane (95). This process does not involve specific plasma membrane receptors. Upon binding of the cationic compound to the plasma membrane, endocytosis occurs, followed by the formation of endosomes. However, vesicular transport is actively downregulated in the BBB to protect the brain from nonspecific exposure to polycationic compounds. Therefore, forcing drugs to enter the brain by absorptive-mediated transcytosis goes against the neuroprotective barrier function, as has been demonstrated for anionic and cationic nanoparticles that disrupted the BBB (96).

Carrier-mediated transcytosis is used for the delivery of nutrients, such as glucose, amino acids, and purine bases, to the brain (97, 98). At least eight different nutrient transport systems have been identified, with each transporting a group of nutrients of the same structure. Carrier-mediated transcytosis is substrate selective and the transport rate is dependent on the degree of occupation of the carrier (99). Therefore, only drugs that closely mimic the endogenous carrier substrates will be taken up and transported into the brain.

In contrast, receptor-mediated transcytosis enables larger molecules, such as peptides, proteins, and genes, to specifically enter the brain. Classical examples of

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receptors involved in receptor-mediated transcytosis are the insulin receptor (100); the transferrin receptor (101, 102); and the transporters for low-density lipoprotein (103), leptin (104), and insulin-like growth factors (105).

Besides many influx mechanisms, several efflux mechanisms exist at the BBB as well. The best known is P-glycoprotein (Pgp) (26–29, 106). Pgp is a transmembrane protein, located at the apical membrane of the BCEC. It has a high affinity for a wide range of cationic and lipophilic compounds and therefore limits the transport to the brain of many drugs, including cytotoxic anticancer drugs, antibiotics, hormones, and HIV protease inhibitors (107). Other MDR efflux mechanisms at the BBB include MDR-related proteins (MRP), such as MRP 1, 2, 5, and 6 (108). In addition, as mentioned above (see section on Blood-Brain Barrier), many other transporters are present at the BBB, such as the organic anion transporter (influx and efflux), the organic cation transport system (influx), and the nucleoside transporter system (influx) (28, 29).

Research over the years has shown that the BBB is a dynamic organ that combines restricted diffusion to the brain for endogenous and exogenous compounds with specialized transport mechanisms for essential nutrients.

Global Brain Delivery by Enhanced Passive Vascular Drug Delivery

Large molecules (e.g., drug conjugates) or drug delivery systems (nano-sized carrier systems, including polymers, emulsions, micelles, liposomes, and nanoparticles) may reach the brain by passive targeting similar to small molecules, meaning that their distribution is mainly determined by physico-chemical and physiological conditions (109–110). Often this results in a very poor distribution into the brain. However, enhanced distribution may be obtained when the permeability of blood vessels in the target tissue has been increased owing to disease conditions, such as in tumors. Such enhanced permeability and retention has been observed in many cases (112), showing increased accumulation of drug in those (mostly peripheral) areas. Unfortunately, drug delivery to the brain by enhanced permeability and retention has not been shown to be effective in treating human brain diseases. Interestingly, recently it was demonstrated in mice that intravascular long-duration delivery of high-dose recombinant human β-glucuronidase to treat the lysosomal storage disease mucopolysaccharidosis VII resulted in decreased lysosomal storage in parietal-neocortical and hippocampal neurons, in glial cells, and in the meninges and perivascular tissue (113). It was suggested that this enhanced uptake had occurred by an extracellular pathway that also allows small amounts of large molecules, such as serum albumin, to enter the brain (114). An additional explanation may be that glycosylated enzymes, such as β-glucoronidase, have, in addition to reduced immunogenicity, improved metabolic stability and a relatively long elimination half-life that favors a long exposition to the BBB, possibly resulting in an increase in brain uptake (115). However, there is currently no experimental evidence that these glycosylated proteins, which have been shown to be active in the brain, are taken up by transporters at the BBB.

Enhanced drug delivery to the brain has also been achieved in various other ways. One is the osmotic disruption/shrinking of the BBB by intracarotid administration of

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a hypertonic mannitol solution. By subsequent administration of drug, substantially increased concentrations can be achieved in brain or tumor tissue. However, because the BBB is temporarily opened under such conditions, which has to be done under anesthesia, it has the disadvantage that neurons may be damaged permanently owing to unwanted blood components entering the brain (116). Therefore, this procedure only has applicability in life-threatening brain diseases [tumors (50)].

A less aggressive approach seems to be the intracarotid administration of alkylglycerol (117), which enhances mainly drug transport by the paracellular route. In rats with brain-implanted C_6-glioma, increased tissue fluorescence was found in both tumor tissue and brain surrounding the tumor following coadministration of FITC-dextran (40 kDa). Similar data in brain tissue were observed following coadministration of methotrexate to nude mice. However, with this type of administration, it appears necessary to use a general anesthetic, which limits its applicability to life-threatening brain diseases such as brain tumors. In addition, no human data are available yet.

Another procedure is the application of a bradykinin-analogue (RMP-7), which has been shown to increase BBB permeability by opening the tight-junctions via a receptor-mediated mechanism, resulting in an enhanced uptake of carboplatin in C_6-glioma implanted in rat brain (118). However, a Phase II study did not show improved carboplatin efficacy in combination with RMP-7 (119). In addition, a Phase III study with RMP-7 and carboplatin with radiotherapy was stopped because of no increase in efficacy.

A general concern about these agents is the possibility that besides opening of the BBB and the associated brain disturbances, application in the treatment of brain tumors could also enhance tumor dissemination via vascular spread of tumor cells.

Enhanced delivery can also be achieved by application of so-called protein transduction domains (PTDs; for reviews see References 120, 121). These PTDs are typical amino acid sequences that are capable of enhancing the delivery of large molecules into cells. Typical examples are the Tat-PTD (122), the homeodomain of Antennapedia (123), SynB-vectors (124), and others (125, 126). These peptides are basic molecules, and therefore cations, that are able to enhance protein uptake by cells mainly by increased adsorptive mediated endocytosis. Toxicity profiles of the various PTDs are an important issue (127), but because this approach lacks cell or tissue selectivity it seems to be particularly suitable for local drug delivery. Clinical proof of principle has recently been demonstrated with SynB-vectors to enhance the transport of morphine-6-glucuronide to the brain (**http://www.syntem.com**). Recently, it was shown that the bacterial redox protein azurin could preferentially enhance the entry of glutathione transferase and green fluorescent protein into cancer cells. The authors claim that cellular entry occurred at least partly by receptor-mediated uptake, where the 28–amino acid peptide acted as a PTD (128).

Therapeutic gene silencing of apolipoprotein-B has been demonstrated in mice following administration of a high dose (50 mg/kg) of modified siRNAs (129). This was achieved by coupling cholesterol to siRNA. Increased uptake by liver, heart, kidney, adipose, and lung, but not by brain, tissue was demonstrated. The uptake into these tissues can be explained by the lipophilization of the siRNA by cholesterol

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and the resulting increase in plasma residence time owing to an increase in plasma half-life from 6 min (naked siRNA) to 95 min (chol-siRNA).

A similar approach has been applied by coupling lipophilic and amphiphilic groups (stearoyl groups or Pluronic block copolymers) to polypeptides to enhance the uptake of these compounds by the brain. However, no selectivity in brain uptake has been demonstrated by applying such modifications (130).

All these approaches are able to more or less enhance the uptake of small and/or large molecules into various tissues, but they lack the selective/specific homing devices needed to target drugs to the brain and to selectively/specifically increase drug delivery to the brain.

Global Brain Delivery by Active Vascular Receptor-Mediated (Nonviral) Drug Targeting

Active physiological or disease-induced drug targeting strategies involve the application of a homing device or technology that utilize endogenous transport mechanisms for site-selective/specific delivery in the body. With respect to the BBB, this involves receptor-mediated transcytosis systems at the BBB to reach extracellular or intracellular targets in the brain. The advantage of active targeting is the increase of the amount of drug in the target tissue, thereby increasing the pharmacological response and reducing systemic side effects. However, when the homing device is not specific, side effects can occur. Interestingly, targeting efficiency can be increased when the target is disease induced, such as occurs for the diphtheria toxin receptor under inflammatory disease conditions (see below). These homing devices can be applied to target biopharmaceutical drugs in conjugates, liposomes, or polymer systems to the brain (8, 131, 132).

The focus of the following paragraphs is on active targeting by receptor-mediated transcytosis, and several examples of receptor-mediated transcytosis that have successfully been employed to target drugs to the brain are reviewed.

In general, receptor-mediated transcytosis occurs in three steps: receptor-mediated endocytosis of the compound at the luminal (blood) side, movement through the endothelial cytoplasm, and exocytosis of the drug at the abluminal (brain) side of the brain capillary endothelium (133). Upon receptor-ligand internalization, clathrin-coated vesicles are formed, which are approximately 120 nm in diameter (134, 135). These vesicles may transport their content to the other side of the cell or go into a route leading to protein degradation. Indeed, at least two important routes for degrading proteins have been identified, including the lysosomal and the ubiquitin-proteasome route (18, 136). Therefore, to escape from the endosomal/lysosomal system, mechanisms have been applied to ensure release of the drug into the cytosol. These include the application of pH-sensitive liposomes (131) or cationic molecules (137). The diphtheria toxin that is also applicable as a targeting ligand, and is discussed later, has an intrinsic lysosomal escape mechanism (138). Nevertheless, with or without application of lysosomal escape mechanisms, protein delivery to the brain has been shown to be effective. Therefore, receptor-mediated transcytosis allows the

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specific delivery/targeting of larger drug molecules or drug-carrying particles (such as liposomes, polymer systems, nanoparticles) to the brain.

TfR: transferrin receptor

Transferrin receptor. The most widely characterized receptor-mediated transcytosis system for the targeting of drugs to the brain is the transferrin receptor (TfR). TfR is a transmembrane glycoprotein consisting of two 90 kDa subunits. A disulfide bridge links these subunits, and each subunit can bind one transferrin molecule (139). The TfR is expressed mainly on hepatocytes, erythrocytes, intestinal cells, and monocytes, as well as on endothelial cells of the BBB (140). Furthermore, in the brain, the TfR is expressed on choroid plexus epithelial cells and neurons (139). The TfR mediates cellular uptake of iron bound to transferrin.

Drug targeting to the TfR can be achieved by either using the endogenous ligand transferrin or by using an antibody directed against the TfR (OX-26 antirat TfR). Each of these targeting vectors has its advantages and disadvantages. For transferrin, the in vivo application is limited due to high endogenous concentrations of transferrin in plasma and the likely overdose of iron when one tries to displace the endogenous transferrin with exogenously applied transferrin-containing systems. However, recent studies in our group (141) have shown that liposomes tagged with transferrin are suitable for drug delivery to BBB endothelial cells in vitro, even in the presence of serum. OX-26 does not bind to the transferrin-binding site and is therefore not displaced by endogenous transferrin.

The TfR is responsible for iron transport to the brain. So far, the intracellular trafficking of transferrin and OX-26 upon internalization via the TfR has not yet been elucidated. Some reports suggest transcytosis of transferrin across the BCEC, whereas others claim endocytosis of transferrin, followed by an intracellular release of iron and a subsequent return of apotransferrin to the apical side of the BCEC (97, 143). Moos & Morgan (2000) have shown that the transcytosis of iron exceeds the transcytosis of transferrin across the BBB, supporting the second theory (139). Furthermore, these authors have proposed a new theory in which the TfR-transferrin complex is transcytosed to the basolateral side of the BCEC, where transferrin remains bound to the TfR but iron is released into the brain extracellular fluid (143). Subsequently, apotransferrin bound to the TfR will recycle back to the apical side of the BBB. This theory is supported by data from Zhang & Pardridge, who found a 3.5-fold faster efflux from brain to blood of apotransferrin than holo-transferrin (142). In addition, in a recent publication, Deane et al. illustrated that free iron is rapidly taken up by brain capillaries and subsequently released into the brain extracellular fluid and CSF at controlled moderate to slow rates (144).

The mechanism of transcytosis of OX-26 is not yet fully elucidated. Pardridge and colleagues have shown efficient drug targeting and delivery to the brain in vivo by applying OX-26 (examples can be found in References 97, 133). In contrast, Broadwell et al. (1996) have shown that both transferrin and OX-26 are able to cross the BBB, but that the transcytosis of transferrin is more efficient (145). Furthermore, Moos & Morgan have shown that OX-26 mainly accumulates in the BCEC and not in the postcapillary compartment (146). In addition, iron deficiency did not increase OX-26 uptake in rats. Our data, as well as literature reports, show that iron deficiency causes

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an increase in TfR expression (139, 147). Therefore, it is expected that the uptake of OX-26 would increase as well. The data by Moos & Morgan suggest that OX-26 transcytosis might result from a high-affinity accumulation by the BCEC, followed by a nonspecific exocytosis at the basolateral side of the BCEC (146). In addition, these authors found a periventricular localization of OX-26, which suggests that OX-26 probably also is transported across the BCSFB.

Although the mechanism of transcytosis of transferrin and OX-26 is not yet fully elucidated, it is important to realize that drug delivery to the brain via the TfR has been shown to be possible. By this approach, vasoactive intestinal peptide (VIP), BDNF, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and peptide nucleic acids, as well as pegylated immunoliposomes containing plasmid DNA encoding for beta-galactosidase, tyrosine hydroxylase and short hairpin RNAs, have all been transported into the brain parenchyma (148). However, OX-26 is an antibody against the rat TfR and does not bind to the human TfR, making it impossible to translate this technology to the clinic. Moreover, unless they are humanized, rat antibodies will cause immunogenic reactions in humans. The preparation of humanized or chimeric antibodies is difficult, and in some cases this may lead to a loss of affinity for the target receptor. In addition, one can argue that the administration of antibodies directed against such an important uptake mechanism involved in iron homeostasis poses a risk for human application.

Preferably, a targeting vector directed to the TfR would be small, nonimmunogenic, and should initialize internalization of the TfR upon binding. Xu et al. have used a single-chain antibody Fv fragment against the human TfR, which was tagged with a lipid anchor for insertion into a liposomal bilayer (149). The molecular weight of this antibody fragment, including the lipid anchor, was approximately 30 kDa. In addition, Lee et al. have used a phage-display technique to find small peptide ligands for the human TfR (150). They obtained a 7- and a 12-mer peptide that bind to a different binding site than transferrin and are internalized by the TfR. Although these small peptides can also exert immunogenic reactions in humans, they are promising ligands for drug targeting to the human TfR on the BBB.

Insulin receptor. Another widely characterized, classical, receptor-mediated transcytosis system for the targeting of drugs to the brain is the insulin receptor. Again, just as for the TfR system, Pardridge and colleagues have documented the use of the insulin receptor for the targeted delivery of drugs to the brain (148).

The insulin receptor is a large 300 kDa protein and is a heterotetramer of two extracellular alpha and two transmembrane beta subunits. Each beta chain contains tyrosine kinase activity in its cytosolic extension. The alpha and beta subunits are coded by a single gene and are joined by disulfide bonds to form a cylinder. Primarily, insulin binds and changes the shape of the receptor to form a tunnel, allowing entry of molecules such as glucose into the cells. The insulin receptor is a tyrosine kinase receptor and induces a complex cellular response by phosphorylating proteins on their tyrosine residues. The binding of a single insulin molecule into a pocket created by the two alpha chains effects a conformational change in the insulin receptor so that the beta chains approximate one another and it carries out transphosphorylation on

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tyrosine residues. This autophosphorylation is necessary for the receptor to internalize into endosomes. The endosomal system has been shown to be a site where insulin signaling is regulated, but also a site where the degradation of endosomal insulin occurs. Most of the insulin is degraded, but less so in endothelial cells (151), whereas the receptors are largely recycled to the cell surface. Endocytosis is not necessary for insulin action, but probably is important for removing the insulin from the cell so the target cell for insulin responds in a time-limited fashion to the hormone. This endocytosis mechanism of the insulin receptor has been exploited for the targeting of drugs to the brain.

As for transferrin, the in vivo application of insulin as the carrier protein is limited, mainly owing to the high concentrations of insulin needed and the resulting lethal insulin overdosing. Therefore, drug or gene delivery to, for instance, rhesus monkeys is performed with the murine 83-14 MAb that binds to the exofacial epitope on the alpha subunit of the human insulin receptor. In the primate, the MAb has a BBB permeability surface area (PS) product that is ninefold greater than murine MAbs in the human TfR (152). Using this MAb, Pardridge and coworkers have successfully made radiolabeled amyloid-β peptide$_{1-40}$ (Aβ_{1-40}), serving as a diagnostic probe for Alzheimer's disease, and they have pegylated immunoliposomes containing plasmid DNA encoding for beta-galactosidase available in the brain of primates (152).

Unfortunately, the 83-14 MAb cannot be used in humans owing to immunogenic reactions to this mouse protein. However, genetically engineered, effective forms of the MAb have now been produced, which may allow for drug and gene delivery to the human brain (153). Still, one can argue that the administration of antibodies directed against such an important mechanism involved in glucose homeostasis poses a risk for human application.

LRP1 and LRP2 receptor. During the past few years, the LRP1 and LRP2 (also known as megalin or glycoprotein 330) receptors have been exploited to target drugs to the brain in a similar fashion as the transferrin and insulin receptors. Both LRP1 and LRP2 receptors belong to the structurally closely related cell surface LDL receptor gene family. Both receptors are multifunctional, multiligand scavenger and signaling receptors. A large number of substrates are shared between the two receptors, like lipoprotein lipase (LPL), α2-macroglobulin (α2M), receptor associated protein (RAP), lactoferrin, tissue- and urokinase-type plasminogen activator (tPA/uPA), plasminogen activator inhibitor (PAI-1), and tPA/uPA:PAI-1 complexes. More specific ligands for the LRP1 receptor are, for example, melanotransferrin (or P97), thrombospondin 1 and 2, hepatic lipase, factor VIIa/tissue-factor pathway inhibitor (TFPI), factor VIIIa, factor IXa, Aβ_{1-40}, amyloid-β precursor protein (APP), C1 inhibitor, complement C3, apolipoproteinE (apoE), pseudomonas exotoxin A, HIV-1 Tat protein, rhinovirus, matrix metalloproteinase 9 (MMP-9), MMP-13 (collagenase-3), spingolipid activator protein (SAP), pregnancy zone protein, antithrombin III, heparin cofactor II, α_1-antitrypsin, heat shock protein 96 (HSP-96), and platelet-derived growth factor (PDGF, mainly involved in signaling) (154–156), whereas apolipoproteinJ (apoJ, or clusterin), Aβ bound to apoJ and apoE, aprotinin, and very-low-density lipoprotein (VLDL) are more specific for the LRP2 receptor (157, 158).

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Béliveau's group first reported that melanotransferrin/P97 was actively transcytosed across the BBB and suggested that this was mediated by the LRP1 receptor (156). Melanotransferrin is a membrane-bound transferrin homologue that can also exist in a soluble form and is highly expressed on melanoma cells compared with normal melanocytes. Intravenously applied melanotransferrin delivers the majority of its bound iron to the liver and kidney, where only a small part is taken up by the brain (159). After conjugation to melanotransferrin, the Béliveau's group was able to successfully deliver doxorubicin to brain tumors in animal studies (160). This melanotransferrin-mediated drug-targeting technology (now designated NeuroTrans™) is currently being developed by BioMarin Pharmaceuticals Inc. (Novato, CA) for the delivery of enzyme replacement therapies to the brain. Interestingly, together with researchers from BioMarin, Pan et al. recently reported the efficient transfer of RAP across the BBB by means of the LRP1/LRP2 receptors, suggesting a novel means of protein-based drug delivery to the brain (161). RAP is a 39 kDa protein that functions as a specialized endoplasmic reticulum chaperone assisting in the folding and trafficking of members of the LDL receptor family. In unpublished results, the Béliveau group have now filed a patent application on the use of the LRP2-specific ligand aprotinin, and more specifically on functional derivatives thereof (e.g., angio-pep1), thereby providing a noninvasive and flexible method and carrier for transporting a compound or drug across the BBB (162). Aprotinin (Trasylol®) is known as a potent inhibitor of serine proteases such as trypsin, plasmin, tissue, and plasma kallikrein, and it is the only pharmacologic treatment approved by the U.S. Food and Drug Administration to reduce blood transfusion in coronary artery bypass grafting (163).

In addition to being a tumor marker protein, melanotransferrin is also associated with brain lesions in Alzheimer's disease and is a potential marker of the disorder (164). In addition, the proposed receptor for melanotransferrin, LRP1, has genetically been linked to Alzheimer's disease and may influence APP processing and metabolism and Aβ uptake by neurons through α2M (α2M is one of the Aβ carrier proteins next to, for example, apoE, apoJ, transthyretin, and albumin) (157). Furthermore, a close relationship with RAGE (receptor for advanced glycation end products) in shuttling Aβ across the BBB has been described. In addition, the LRP2 receptor has also been described to mediate the uptake of Aβ complexed to apoJ and apoE across the BBB (157). This complex interaction with Alzheimer's disease makes the safety of using LRP1/LRP2 receptors for the targeting of drugs to the brain difficult to predict in humans, especially when the complex signaling function of these receptors is included in the assessment [for example, the control of permeability of the BBB, vascular tone, and the expression of MMPs (22)], as well as the fact that both the receptors are critically involved in the coagulation-fibrinolysis system. In addition, melanotransferrin was also reported to be directly involved in the activation of plasminogen (165), and high plasma concentrations of melanotransferrin are needed to deliver drugs to the brain, perhaps resulting in dose limitations because of the high iron load in the body.

The same line of reasoning for the interactions at the level of the uptake receptors may apply to the use of RAP and aprotinin (derivatives). On the other hand, the latter has already been successfully tried in humans, usually without severe side effects,

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indeed making the peptide derivatives potentially safe drug carriers. As for RAP, no results on the efficacy or capacity of the aprotinin peptides as carrier for drugs are yet available.

DTR: diphtheria toxin receptor

Diphtheria toxin receptor. Recently, our group has identified a novel human applicable carrier protein (known as CRM197) for the targeted delivery of conjugated proteins across the BBB (166). Uniquely, CRM197 has been used for a long time as a safe and effective carrier protein in human vaccines, and recently as a systemically active therapeutic protein in anticancer trials (167, 168). This has resulted in a large body of prior knowledge on the carrier protein, including its transport receptor and mechanism of action, receptor binding domain, conjugation- and manufacturing process, and kinetic and safety profile in animals and humans. CRM197 delivers drugs across the BBB by the well-characterized, safe, and effective mechanism called receptor-mediated transcytosis (see **Figure 5**). From the literature, it was already known that CRM197 uses the membrane-bound precursor of heparin-binding epidermal growth factor–like growth factor (HB-EGF) as its transport receptor (138). This precursor is also known as the diphtheria toxin receptor (DTR). In fact, CRM197 is a nontoxic

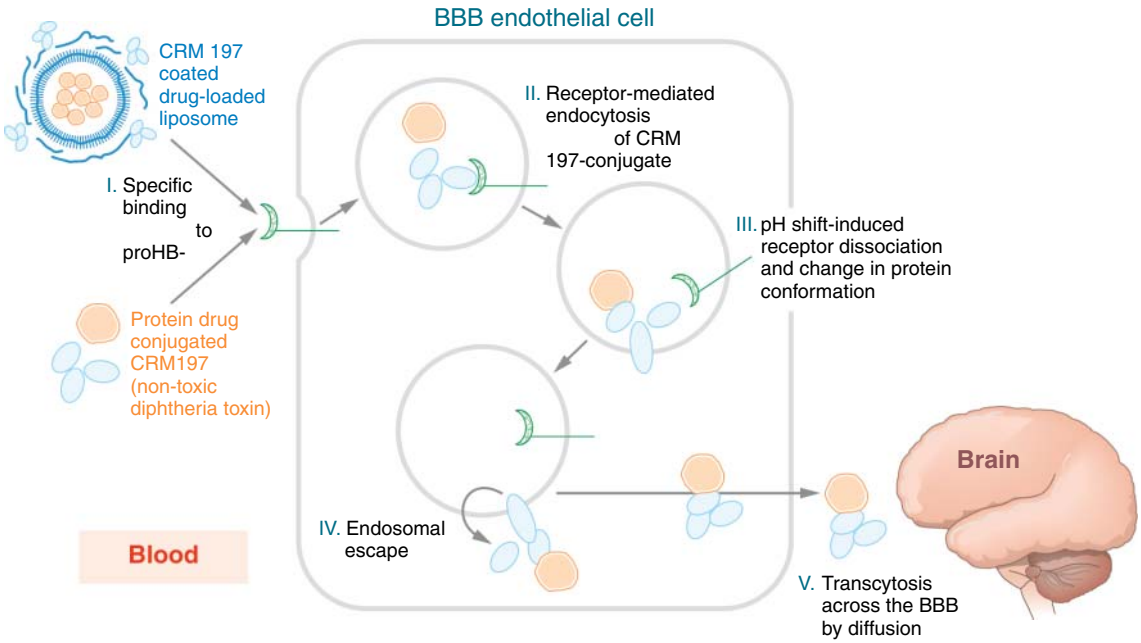

Figure 5

An illustration of CRM197-targeted and diphtheria toxin receptor–mediated uptake of CRM197 conjugates and liposomes by blood-brain barrier endothelial cells. Following internalization, the systems are subsequently released from the endosomal/lysosomal compartment into the cytosol of the cell by an intrinsic endosomal escape mechanism of the CRM197. The (conjugated) drug is released at the brain side, most likely by a nonspecific exocytosis mechanism.

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mutant of diphtheria toxin. Membrane-bound HB-EGF is constitutively expressed in the BBB, neurons, and glial cells (169). HB-EGF expression is strongly upregulated in the cerebral blood vessels by, for instance, ischemic stroke and in gliomas (170, 171), which may lead to a site-selective improvement of the therapeutic efficacy of the targeted drugs in the brain.

Brain drug targeting: directing drugs specifically to the brain

One of the remarkable features of the diphtheria toxin (including CRM197) is its evolved intrinsic endosomal escape mechanism, which allows the protein to enter the cytosol of the cell bypassing the lysosomal degradation system (195, 196). This provides interesting opportunities for the efficient intracellular delivery/targeting of biopharmaceuticals such as enzymes, RNAi, and genes. By means of the dynamic cell culture model of the BBB, our group was able to demonstrate the functional expression of the DTR, safety of the CRM197 carrier protein, and specific transport efficacy of CRM197 carrier protein conjugates to a 40 kDa enzyme (horseradish peroxidase, HRP, serving as a model protein drug) and DTR-targeted pegylated liposomes containing HRP. In addition, the in vivo proof-of-principle with this novel brain drug targeting technology was demonstrated by the specific brain uptake of DTR-targeted HRP in guinea pigs (166).

Although HB-EGF is expressed with a similar tissue distribution in many species, including human, monkey, rat, and mouse, only rat and mice are resistant to diphtheria toxin because of an amino acid substitution in the receptor-binding domain on HB-EGF that reduces binding of diphtheria toxin to rodent HB-EGF. Fortunately, a transgenic mouse conditionally expressing the human DTR was recently generated by Cha and coworkers (172), allowing specific study of brain drug delivery technology in mice.

Another known complication of the bacterial CRM197 protein is that neutralizing antibodies against diphtheria toxin may develop or already be present in serum of the recipient because of earlier vaccinations, thereby reducing the efficacy of the drug delivery system. There are, however, several lines of evidence that such an immune response to CRM197 can occur, but it may not be a problem in the clinic, at least not for the treatment of acute indications. The clinical studies performed by Buzzi et al. indicate that preexisting levels of neutralizing antibodies were actually decreased 30 days after repeated treatment with CRM197 (168). An antibody response to these antigens seems to occur mainly following subcutaneous and intramuscular injection, but seems to be reduced following intravenous administration.

Another interesting aspect of the DTR is that this receptor is strongly upregulated under inflammatory disease conditions (166), such as those occurring in many brain diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, ischemia, encephalitis, epilepsy, tumors, lysosomal storage diseases, etc. This may enhance the therapeutic effect by disease-induced targeting. This could also be used to image (inflammatory) disease areas in the brain. For example, it has already been shown that the DTR is upregulated in the brain following seizures (173). By applying CRM197-coated liposomes loaded with an MRI-enhancing agent (e.g., gadolinium), it would be possible to image such disease areas, as has been done for the glucose receptor (174). This would be particularly useful to investigate the extent of damage/recovery and disease progression/therapy in brain diseases that have been mentioned earlier.

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CONCLUSIONS

There is an unmet need for better treatment of brain diseases. In addition to the currently available small-molecule drugs, there are many promising biopharmaceutical agents that, unfortunately, cannot enter the brain in sufficient quantities to be effective. Therefore, new technologies have to be developed to address this problem. In this review, we have highlighted the current research that is underway to improve drug delivery and targeting of biopharmaceutical agents (e.g., proteins, RNAi, and genes) to the brain. The development of technologies to actively target such agents to the diseased brain will open a wide area for diagnostic investigations, the possibility of monitoring disease progression, and the treatment of diseases involving the brain.

With respect to the therapy of brain diseases, the only technology applied thus far to deliver biopharmaceutical agents in humans to the brain is by viral delivery. Viral delivery to the brain has only been performed by direct injection/infusion into the brain because of insufficient brain targeting owing to the lack of a homing process, which makes the viral systems only suitable for local delivery. Therefore, the most promising technology in our opinion is active vascular nonviral receptor-mediated targeting. Vascular nonviral active targeting has been shown to be effective and selective for global delivery of drugs to the brain in several animal models. By using antibodies against receptors (e.g., transferrin- and insulin receptor), proteins (enzymes), RNAi, and genes could be targeted to the brain. However, these applications cannot be directly transferred to humans. First, because the antibodies used in animals cannot (without humanization) directly be applied in humans. Second, antibodies are meant to bind to their receptor, which means that administration of these will reduce the availability of receptors at the BBB and in the brain. Moreover, several of these receptors (insulin receptor and the multiligand LRP1 and LRP2 receptors) are involved in cell signaling processes and, therefore, brain targeting by such antibodies may interfere with the transport of endogenous ligands (e.g., insulin, transferrin) and brain signaling. They also cause downregulation of receptors and should therefore not be used for chronic administration. The melanotransferrin receptor seems to be involved in the release of plasmin (165) and, therefore, with the blood clotting cascade, and the LRP1 receptor seems to be involved with apoliprotein J transport in a complex with Alzheimer's amyloid-beta (175). Interference with such transport systems poses a potential hazard. The DTR seems to be a safe and effective uptake receptor for the targeting of drugs to the brain that may be applicable to humans. Moreover, the DTR does not have an endogenous ligand, so there is no competition for transport. Furthermore, CRM197 has already been safely tried in humans. However, even though specific brain uptake of a DTR-targeted enzyme has been demonstrated in guinea pigs, this approach now awaits further in vivo validation in terms of kinetics of brain distribution and efficacy of targeted drugs in relevant disease models of the CNS.

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DISCLOSURE STATEMENT

Dr. Gaillard is the inventor of a patent on the technology described in this review and is the director and a shareholder of the company that owns this patent.

Dr. de Boer is an Associate Professor of Pharmacology at the Leiden-Amsterdam Center for Drug Research at Leiden University, the Netherlands; cofounder and shareholder of To-BBB Technologies BV; and Chairman of the Scientific Advisory Board of To-BBB Technologies BV. Research on drug delivery in lysosomal storage diseases is funded by the Sanfilippo Foundation (U.S.A.); research on drug delivery in ischemia is funded by the Stichting Technische Wetenschappen (the Netherlands); and research on drug delivery in Alzheimer's disease is funded by the Internationale Stichting Alzheimer Onderzoek (the Netherlands). Dr. de Boer is the coinventor of a patent on drug targeting to the brain: Patent application (filed 10-02-03): Differentially expressed nucleic acids in the blood-brain barrier under inflammatory conditions.

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Errata

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