Drug targeting to the diseased brain

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Abstract

The delivery of hydrophilic drugs to the brain is limited by the various barriers in the brain. In this respect, the blood-brain barrier (BBB) is the most important barrier because it functions as a physical and metabolic barrier represented by the endothelial cells of brain capillaries. In order to treat brain diseases, drugs must pass the BBB by targeting them to internalizing transport systems at the BBB to overcome the BBB. In this paper, we summarize some approaches that may be successful for clinical application. The applicability of Cross Reacting Material (CRM)197 as a receptor-mediated targeting agent - particularly under disease induced circumstances - seems favorable and effective.

Keywords: blood-brain barrier, receptor-mediated drug targeting, disease-induced drug targeting

1 Barriers in the brain

There are three barriers that limit drug transport to the brain parenchyma: the blood-brain barrier (BBB) localized in the capillaries in the brain; the blood-cerebrospinal-fluid barrier (BCSFB) which is presented by the choroid plexus (CP) 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 (de Boer and Gaillard, 2007). In this respect, the BBB is the most important barrier for drugs entering the brain from the blood side, since the surface of the BBB is facing the blood and that of the BCSFB and the ependyma the ventricle.

1.1 BBB protective mechanisms

The concept of the BBB has been developed from experiments by Ehrlich (1885) and Goldman (1913) and has later also been demonstrated histologically by electron-microscopy. The BBB is presented by brain capillary endothelial cells (BCECs) in conjunction with astrocytes (Fig. 1).

Fig. 1. Strategies for blood-brain barrier drug transport (modified from Abbott and Romero, Mol. Med. Today, 1996).

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Other cell types such as pericytes and neuronal cells also play a role in BBB functioning as a tight barrier limiting the transport of hydrophilic drugs. Therefore, paracellular transport does not occur across an intact BBB. Next to its physical barrier properties, various transport systems have been discovered that play an important role in maintaining brain homeostasis and BBB integrity. These may also influence (increase and/or decrease) drug transport to the brain. Transepithelial transport can occur by diffusion of lipophilic compounds, adsorptive-mediated endocytosis (pinocytosis), and by influx and efflux transporters that regulate the entry of drugs into the BCECs, presenting the BBB, and eventually into the brain (de Boer et al., 2003). The influx and efflux transporters comprise carrier-mediated transport systems, including cationic and anionic influx and efflux systems such as P-glycoprotein (P-gp), multidrug-resistance proteins (MRPs), nucleoside transporters, organic anion transporters, organic cation transporters, large amino acid transporters, and the receptor-mediated transport systems such as the transferrin-1 and -2 receptors, the melanotransferrin receptor, and the scavenger receptors SR-AI and SR-BI. Furthermore, other transporters such as peptide and cytokine transporters are operational at the BBB. Since carrier mediated transport systems are suitable for the transport of small molecular compounds into the BBB/brain, we will focus in this paper on receptor-mediated brain targeting. Here we will focus on physiological targeting strategies. Several methods have been described to transport drugs to the brain. These can be divided in three different approaches:

- local delivery by direct injection,
- induction of enhanced BBB permeability
- physiological targeting strategies.

1.3 Receptor-mediated brain targeting

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1.2 BBB intracellular protective mechanisms

The intracellular metabolic activity of the BCECs also forms a part of the barrier. Following endocytosis into BCECs, compounds can follow various intracellular routes that may influence their availability in the BBB/brain. Upon receptor-mediated internalization, clathrin- and non-clathrin-coated vesicles of approximately 120 nm in diameter are formed (Conner and Schmidt, 2003). These vesicles may transport their contents to the other side of the cell followed by exocytosis, or may go into a pathway that leads to protein and DNA/RNA degradation. Indeed, at least two important routes for degrading proteins have been identified including the lysosomal and the ubiquitin-proteasome route (Ciechanover, 2005). For DNA/RNA there may also be endosomal/lysosomal breakdown. DNAses, RNAses (including RISC + endo- and exonucleases) may add to the breakdown of these molecules. These processes will lead to a reduced intracellular availability of proteins, (si)RNA and genes, and consequently, a diminished transport further into the brain.

Therefore, receptor-mediated transport systems that do not follow the degradation pathways following internalization, or those that can escape endosomal or lysosomal degradation should particularly be applied for drugs targeting the BBB/brain.
erythrocytes, intestinal cells, and monocytes. The TIR is furthermore present on endothelial cells of the BBB and on choroid plexus epithelial cells and neurons in the brain. Various methods for targeting drugs to the TIR have been described. An antibody directed against this receptor (OX-26), the endogenous TIR ligand transferrin, and TIR-binding peptides can be used as carrier molecules to target the TIR. All these different approaches have advantages and disadvantages as reviewed previously (Pardridge, 2007). These approaches have been shown effective in in vitro studies and in animal studies treating several brain diseases with large molecular drugs in combination with several targeting devices such as protein conjugates and liposomes. Despite the potency of TIR-mediated delivery no effective clinical application has been developed so far.

![Diagram of blood-brain barrier and drug targeting](image)

**Fig. 2.** Targeting drugs to the BBB and the brain.

**Table 1.** Receptor-mediated targeting strategies that have been used for delivery of drugs across the BBB.

<table>
<thead>
<tr>
<th>Target receptor/transporter</th>
<th>Substrate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin receptor (TFR)</td>
<td>Fe-Transferrin, Mab</td>
<td>Pardridge, 2002</td>
</tr>
<tr>
<td>Insulin receptor (INSR)</td>
<td>Insulin, HIRMab</td>
<td>Pardridge, 2007</td>
</tr>
<tr>
<td>Lipoprotein receptor-related protein (LRP1 and LRP2)</td>
<td>Lactoferrin, ApoE, melanotransferrin, Angiopep®</td>
<td>Regina et al., 2008; Fillebeen et al., 1999</td>
</tr>
<tr>
<td>Diphtheria toxin receptor (DTa)</td>
<td>Diphtheria Toxin, CRM197</td>
<td>de Boer and Gaillard, 2007</td>
</tr>
</tbody>
</table>

**Insulin receptor**  
Pardridge et al. (2007) have applied the insulin receptor for BBB targeting. This method has been well characterized and reviewed. Targeting the insulin receptor using insulin itself is not possible since it interferes with endogenous insulin metabolism and high dosages are lethal. Therefore, targeting to the insulin receptor has been performed using monoclonal antibodies (Mab) that bind to an exofacial epitope of the insulin receptor. Upon binding, the receptor-Mab complex is internalized. Murine antibodies against the human insulin receptor have been used in primates to diagnose Alzheimer’s disease using an amyloid-B peptide, and have furthermore been used to target plasmid DNA to the brain. For human application a humanized antibody has been developed and tested in several studies in primates. Transport
of radioactive therapeutic proteins over the BBB has been shown. So far, however, no therapeutic effects after systemic administration in disease models have been demonstrated. The same targeting device can be used to target genes to the brain using Trojan horse liposomes. Transgene expression has been observed following delivery in brain tissue in diseased and healthy brain but so far no effective clinical application has been developed.

Lipoprotein receptors

Lipoprotein receptor-related proteins LRP1 and LRP2 are related to the cell surface low-density lipoprotein receptor (LDLr). All these receptors are expressed on the BBB and are multifunctional multi-ligand scavenger and signaling receptors (Dehouck et al., 1997). The importance of LRP function in the central nervous system is emphasized by its high expression in the cerebellum, cortex, hippocampus, and brain stem (Bu et al., 1994). Several substrates for this receptor family have been characterized and some of them have been used for strategies to target the brain. Some of these compounds are substrates for both LRP1 and LRP2 while others are more specific for either LRP1 or LRP2.

The transport of lactoferrin across the BBB has been studied. Transcytosis of lactoferrin has been shown LRP-dependent in an in vitro BBB model, and in vivo studies have shown uptake of lactoferrin in the brain (Ji et al., 2006). Secondly, melanotransferrin can be used for receptor-mediated transcytosis across the BBB via the LRP1 receptor. Melanotransferrin is a membrane-bound transferrin homologue that also exists as a soluble compound. Intravenous application of melanotransferrin has resulted in uptake of a small part in the brain and delivery of the majority of the injected dose to the kidney and the liver. Finally, more recent publications describe the selection of Kunitz domain-derived peptides for BBB penetration. One of these peptides (Angiopep-2®) is an effective brain delivery vector. Furthermore, LRP1 has been shown to be involved in the transcytosis process and conjugates of paclitaxel, and angiopep-2 has shown to be active against brain tumors in animal models (Regina et al., 2008). However, recently the collaboration between Angiochem and Geron in the application of LRP1-mediated targeting to tumors has been terminated because of unsuccessful clinical data.

Diphtheria toxin receptor

Diphtheria toxin is taken up by cells via receptor mediated endocytosis (Fig. 3). It uses the membrane-bound precursor of heparin-binding epidermal growth factor (proHB-EGF) as its transport receptor (diphtheria toxin receptor (DT_R)) (Raab and Klagsbrun, 1997). Diphtheria toxin is taken up by cells via receptor mediated endocytosis (Fig. 3). It uses the membrane-bound precursor of heparin-binding epidermal growth factor (proHB-EGF) as its transport receptor (diphtheria toxin receptor (DT_R)) (Raab and Klagsbrun, 1997).
Drug targeting to the brain

DT is not suitable for application as a carrier molecule for targeting the DTₐ because of its toxicity. However, a non-toxic mutant form of DT, called CRM197, is available as a human-applicable carrier protein for targeted delivery to the brain. CRM197 is being used in human vaccines and also as a therapeutic protein to scavenge the soluble form of HB-EGF thereby inhibiting the growth of tumors (Anderson, 1983; Buzzi et al., 2004; Maeda, 2013). Recently, CRM197 has been shown to carry molecules to the BBB both in in vitro and in vivo experiments. At least, reactive astrocytes in lesions of human MS-brains showed a highly induced expression of HB-EGF which offers interesting opportunities for targeted drug treatment (Schenk et al., 2013).

2 BBB/brain targeting and the influence of disease status

BBB protective mechanisms are often less active during diseased states of the brain; therefore, in most brain diseases BBB integrity has changed. This has been described for Alzheimer’s disease, Parkinson’s disease, epilepsy, ischemia, HIV, multiple sclerosis, and other brain diseases. The inflammatory process that is associated with these diseases is common to all of these conditions and is of importance to the nature of the BBB since it is the main cause of a changed functionality/permeability of the BBB.

Next to CNS inflammation, drugs such as glucocorticoids and interferon-alpha and -beta are also able to influence BBB integrity by closing the BBB. This will also influence the functionality/permeability of the BBB and hence drug delivery to the brain (Abbott, 2000; Gaillard et al., 2003). Moreover, the disposition of drugs in the brain (neuropharmacokinetics) under disease conditions may influence the treatment of brain diseases (Reichel, 2006).

Changes in BBB permeability by CNS diseases can be caused by a change in specific receptor-mediated transport or by effects on non-specific transport pathways. Both paracellular transport and transcellular transport such as adsorptive-mediated endocytosis (pinocytosis) or receptor-mediated endocytosis may be increased during disease. Subsequently, this may increase the efficacy of drugs in the brain. Thus, drugs that normally are unable to cross the BBB may reach their target areas in the diseased brain due to this brain ‘enhanced permeability and retention’ (EPR; Maeda, 2013).

2.1 Paracellular transport in disease state

Tight junctions (TJs) are essential for the regulation of brain homeostasis and protect the microenvironment of the brain. In many brain pathologies TJs are disrupted, resulting in increased paracellular transport. Inflammatory conditions present in most brain diseases lead to the production of cytokines that result in TJ disruption. This has been demonstrated in vitro as well as in animal and human studies. An additional mechanism is the rise in intracellular free calcium levels initiated by inflammatory mediators and leading to TJ disruption (Easton and Abbott, 2002).

2.2 Transcellular transport in disease state

2.2.1 Adsorptive mediated endocytosis (pinocytosis)

Cationic (targeting) molecules enter the brain via adsorptive-mediated endocytosis (Kumagai et al., 1987). However, changes in endocytotic activity of the endothelial cells in disease state do not only affect this drug transport route but may also influence the efficacy of other strategies such as receptor-mediated endocytosis. Studies in CNS endothelial cells in vitro have shown an increased adsorptive-mediated endocytosis after TNF-alpha or IL-6 treatment (Duchini et al., 1996), while an increased endocytotic activity has been observed in rats with an occluded middle cerebral artery as a model for brain ischemia (Cipolla et al., 2004).

2.2.2 Receptor mediated endocytosis

Some macromolecules are taken up in the brain via internalizing receptors present on the luminal (blood) side of the brain endothelial cells. Some of these macromolecules can be used as carrier proteins to target drugs to the brain (see paragraph 1.2). Expression of the target receptors is often influenced by the specific disease and the disease state that is being treated. Whether this is favorable or unfavorable for drug targeting depends on the type of receptor targeted, the regulation of its expression, and its brain distribution. Therefore, when applying the targeting strategies mentioned in paragraph 1.2, receptor expression and/or functionality may be changed during disease state.

The TIR also mediates the uptake of iron-bound to transferrin in the brain. A decrease in TIR expression in the hippocampus has been shown in Alzheimer’s disease. Although TIR presence is not studied in all brain diseases, it is clear that the presence of iron in the brain has a pivotal role in progression of brain
diseases. Therefore, iron metabolism may influence the efficacy and safety of TIR-mediated targeting to the brain.

The insulin receptor plays an important role in diabetes and obesity. Expression of the insulin receptor is influenced during these diseases and also during brain diseases such as Alzheimer’s disease (Meissier and Teutenberg, 2005). Targeting the insulin receptor in an attempt to treat a certain disease may result in perturbation of insulin metabolism, may cause insulin resistance, or may influence the efficacy of drugs during brain diseases.

As mentioned before, LRP has a high expression in the cerebellum, cortex, hippocampus, and brain stem (de Boer and Gaillard, 2007). LRP expression has been observed in cells involved in Alzheimer’s disease and tissue repair. The importance of this receptor in brain development has also been shown (Jeynes and Provias, 2008). Although the role and regulation of the LRP receptor is not completely understood, it is very likely that expression of the receptor is influenced during most inflammatory brain diseases. Several studies have shown inhibition of inflammatory processes by LRP (Gaultier et al., 2008).

The expression of the DTR is strongly upregulated under inflammatory disease conditions. These inflammatory conditions occur in many brain diseases including Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, and ischemia. Indeed, it has been shown, by quantifying mRNA levels, that the expression of DTR is increased under disease conditions such as stroke and gliomas (Mishima et al., 1998; Tanaka et al., 1999). The presence of DTR mRNA in rat brain has been shown by in situ hybridization studies following epileptic seizures (Opanashuk et al., 1999). Therefore, receptor upregulation in the diseased area may enhance the therapeutic efficacy and may result in disease-induced targeting. However, two complicating properties of DTR expression and functioning are the expression of co-factors needed for internalization, and the possible protease-mediated shedding of membrane-associated DTR, which may result in reduced availability of the receptor for drug targeting.

2.2.3 Efflux transporters
Active transport mechanisms are important to maintain brain homeostasis. Efflux transporters such as Pgp are present on endothelial cells and transport harmful substrates out of the brain. Transport of drugs (e.g., Pgp substrates) by efflux transporters out of the brain can be influenced by several diseases. Upregulation of Pgp expression in the brain due to inflammatory diseases has been confirmed by several studies. Infectious diseases such as HIV result in upregulation of Pgp expression. Treatment of endothelial cells with HIV-1 TAT results in higher levels of Pgp (Hayashi et al., 2006). This has also been observed in brain slices of patients with HIV encephalitis. Studies in mice with middle cerebral arterial occlusion also reported upregulated Pgp expression in the ischemic area (Spudich et al., 2006). And finally, drug resistance in epilepsy may be due to upregulation of Pgp expression and other drug efflux transporters in the brain (Loscher, 2007).

3. Targeting drugs indirectly to the brain and other tissues
Afergan et al. (2008) have demonstrated the delivery of serotonin to the brain of rats and rabbits following intravenous administration of serotonin-containing liposomes. They suggest that these liposomes are delivered to the brain by monocytes following phagocytosis of the liposomes in the blood compartment. Monocytes are involved in the immune surveillance of the body and are able to pass barriers, including the BBB, to enter sanctuary sites. Schmidt et al. (2003) have also shown delivery of drugs to MS-lesions in the brain following intravenous administration of untargeted liposomes. They also suggest that these were first phagocytosed by monocytes and subsequently delivered to the brain.

Recently we have also demonstrated the uptake of liposomal systems by monocytes following intravenous administration and targeted delivery to the DTR receptor at monocytes in mice with a human immune system (HIS-mice; Fig. 4) and hamsters (Fig. 5) (Schenk et al., 2012). These experiments suggest a way for indirect but actively targeted delivery of drugs via monocytes to the brain, but also to other tissues and sanctuary sites and areas with an inflammatory disease. In addition, the activity of monocytes themselves may be also down- or upregulated in (CNS) diseases (multiple sclerosis, Alzheimer’s disease, Parkinson’s disease, meningitis, encephalitis, (brain) tumors, arthritis, rheuma, etc.). Therefore, the indirect treatment of these conditions following targeted delivery to monocytes may open new ways for therapeutic intervention especially for those diseases that occur at sanctuary sites.
Fig. 4. In-vivo uptake of CRM-FITC and BSA-FITC by tissues and blood cells in mice with a human immune system (HIS-mice) (reproduced with permission of Elsevier; Schenk et al., 2012)

Fig. 5. Uptake of untargeted and targeted liposomes by blood cells (e.g. monocytes) following intravenous administration to hamsters. (reproduced with permission of Elsevier; Schenk et al. 2012)
In conclusion

The blood-brain barrier (BBB) maintains the homeostasis of the brain by regulating particularly the influx/efflux and metabolism of endogenous compounds and exogenous drugs. Intracellular systems are available for the degradation of proteins and DNA/RNA. All these processes protect the brain but are unfavorable for targeting macromolecular drugs across (or to) the BBB. In recent papers, the use of receptor-mediated transcytosis to target the brain has been described (see paragraph 1.2). Although these studies show successful delivery, the efficiency is often low and/or not suitable for human application.

It is well known that the integrity of the BBB is changed under disease conditions. This happens particularly under inflammatory disease conditions that occur in many CNS diseases where BBB functionality (including permeability) has changed (see paragraph 2). This may be due to up-/downregulation of transporters, increased paracellular permeability, and increased pinocytotic activity. The literature clearly shows the impact of different diseases on BBB permeability, and many of these features contribute to the EPR-effect. When studying the action of drugs in the diseased brain this should be taken into account.

Various approaches used to target the diseased brain may fail or be successful because of one or more of the above-mentioned changes in BBB permeability. It is our opinion that the increased endocytic (pinocytotic) transport (e.g., transcytosis) should not be neglected in the evaluation of CNS effects following receptor-mediated targeting of proteins and DNA/RNA to the brain. Possibly, the observed effects in receptor targeting studies are a consequence of the specific targeting molecules that are used, but also of the aforementioned disease conditions-related effects on BBB integrity. Such transport can be very helpful in the therapeutic treatment of CNS diseases and we believe that it is important to investigate the extent to which these altered states of transport contribute to the delivery of drugs to the brain. It is not unlikely that the ability of carrier molecules to cross the BBB is influenced by the progression of the disease that is treated. Using the correct control groups in these studies, and studying both plasma- and neuropharmacokinetics is essential to study drug transport to the brain in a diseased and/or healthy animal.

Chronic treatment of the brain using targeting strategies to receptors may interfere with the endogenous metabolism of the receptors and may result in an immune response to the carrier/macromolecular drug complex. To avoid this problem it seems more feasible to treat brain diseases that need acute treatment such as brain tumors, encephalitis, or stroke) rather than chronic diseases - such as Alzheimer’s disease, epilepsy, and Parkinson’s disease. For chronic treatment of diseases, gene therapy may be an alternative approach since gene therapy ideally results in long-term expression of a transgene that is beneficial to the treatment of the disease.

Degradation of macromolecular drugs after cellular uptake depends on the uptake mechanism and the intracellular pathway of the carrier-drug-receptor complex. The targeting strategies summarized in this review show receptor mediated uptake of drugs and its presence at the luminal side of the BBB. The intracellular pathways may involve escape from the lysosomal degradation system, exocytosis, or endosomal escape followed by diffusion. The exact pathway of degradation of the carrier molecule is for most approaches not completely clear.

In in vitro studies done in our group using DTR targeting, high amounts of drug in the brain capillary endothelial cells were observed, a part of which was able to enter the brain. New strategies that focus on BBB targeting instead of transport across the BBB may be feasible and very useful. First of all, this can be achieved by targeting proteins that have their therapeutic target in the brain endothelial cells. Possible applications are diseases that cause inflammatory conditions in endothelial cells or infectious diseases like encephalitis. An alternative approach is the delivery of genes to the endothelial cells of the BBB. Subsequently, the endothelial cells of the BBB will incorporate the delivered gene and start to produce a therapeutic protein. This can be accomplished by several of the presently available technologies involving receptor-mediated uptake or by exploiting the increased pinocytotic transport that is common to the BBB during disease conditions (described above). Such genes may encode proteins adapted with signaling peptides that target them to specific locations, such as the cell membrane or other intracellular or extracellular targets, depending on the required function and place of action of the encoded protein. This can modify the BBB and make it function like a protein factory that delivers its therapeutic proteins to the brain. This approach can be directly applied to existing (targeting) technologies following intravenous administration. We believe that using the BBB
as a protein factory can lead to an efficient delivery of therapeutic proteins into the brain.

Finally, the indirect and active targeting of drugs to inflammatory disease areas including sanctuary sites, following intravenous administration of CRM197-targeted drugs and liposomes, and subsequently by diphtheria toxin receptor-mediated uptake by monocytes, may represent new ways for the delivery of drugs to sites that can normally not or hardly be reached. Successful clinical application of this and other targeting technologies can hopefully result in better treatment of well-known severe (brain) diseases and make use of an enormous potential of therapeutic macromolecules that are already available.

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