The Transferrin Receptor at the Blood-Brain Barrier - exploring the possibilities for brain drug delivery

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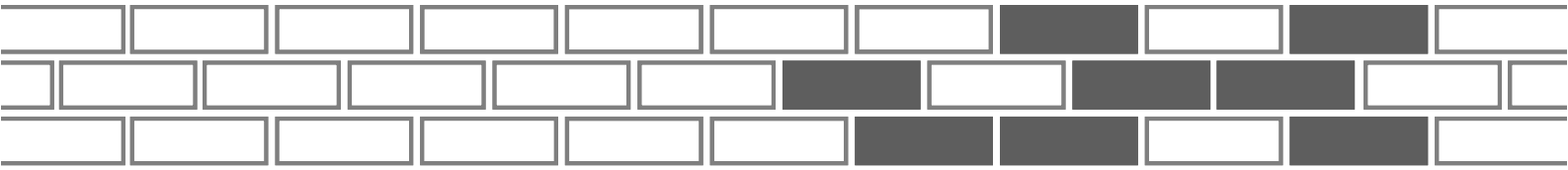
Chapter 8



Summary, discussion and perspectives

Parts of this chapter will be published in an invited review

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Contents Chapter 8

1. Introduction

Drug delivery to the brain is limited due to the presence of the blood-brain barrier (BBB) (1). This barrier is located at the interface between blood and brain and its primary function is to maintain homeostasis in the central nervous system (CNS). Anatomically, the BBB is located at the brain capillary endothelial cells (BCEC), and its unique properties are induced by interactions with astrocytes, pericytes and neuronal cells (2, 3). This makes the BBB a highly dynamic organ.

The research described in this thesis focuses on the transferrin receptor (TfR) at the BBB for drug targeting and delivery to the brain. The TfR is abundantly expressed on BCEC, and is actively internalising its endogenous ligand, transferrin (Tf). For drug delivery purposes Tf-drug conjugates and Tf-tagged liposomes have been studied.

2. The transferrin receptor: target validation

To characterise and validate the TfR for drug targeting to the brain, primary cultured BCEC were used. These BCEC are the main component of the BBB and can be used for in vitro BBB research. To induce BBB-like properties, such as tight junctions and P-glycoprotein expression, BCEC can be co-cultured with astrocytes (4). However, for this research we have cultured the BCEC in astrocyte-conditioned medium (4) to be able to characterise the TfR more mechanistically.

The endogenous ligand, transferrin (Tf), was used for the characterisation of the TfR. To this end Tf was labelled with ^{125}I, to be able to quantify the receptor expression and internalisation (**chapter 3**). Table I summarises the TfR expression levels under physiological conditions (control), as well as in low (DFO) and high ($FeCl_3$) iron situations.

Table I: Total and extracellular expression, determined at 4 °C, of the TfR on primary cultured BCEC and the association of Tf, determined at 37 °C in the presence of DFO or $FeCl_3$ (1 mM). Data were analysed using NONMEM, K_d was estimated 2.4 ± 0.3 μg/ml and 5.0 ± 0.5 μg/ml for expression and association, respectively. Values for B_{max} are summarised in the table as mean (95 % confidence interval, CI). Intra-individual residual variation was determined with a proportional error model and was 10 % for expression levels and 9 % for association.

	B_{max} (fmol/μg cell protein)		
	total TfR expression	extracellular TfR expression	association
control	1.37	0.13	0.90
(CI)	(1.15 – 1.59)	(0.10 – 0.16)	(0.78 – 1.01)
DFO	3.68	0.63	2.31
(CI)	(2.73 – 4.63)	(0.55 – 0.71)	(2.03 – 2.59)
$FeCl_3$	1.64	not detectable	0.56
(CI)	(1.09 – 2.19)		(0.49 – 0.63)

The data in table I were analysed by nonlinear mixed effects modelling, allowing the estimation of a single unique value for the affinity (K_d) in conjunction with separate values for the receptor expression (B_{max}). Approximately 90 % of the receptors is stored in an intracellular pool, as can be seen from the difference between the high total TfR expression and the low extracellular TfR expression. After pre-incubation with the iron scavenger deferoxamine mesylate (DFO), both the expression as well as the association are increased. An excess of $FeCl_3$ has no effect on the total TfR expression, but lowers the association of Tf by the TfR. Therefore, we concluded that the TfR expression level was mainly regulated by the iron concentration in the cell culture medium. This was confirmed by literature reports (5-8). Furthermore, in the presence of the iron scavenger DFO, the increase in extracellular TfR expression was higher than the increase in total TfR expression. This indicates that not only new receptors are synthesised, but that there is also a shift from the intracellular pool to the extracellular membrane. The up-regulation of the TfR expression level constitutes in theory a basis for an increased targeting efficiency to this receptor. DFO (desferal®) is currently used for treatment of (transfusional) iron overload, but it has many side effects (9). *In vivo* upregulation of the TfR may still be possible when new iron chelators are developed. Furthermore, for the treatment of brain cancer, DFO could be used for its antiproliferative effects (10).

To determine the internalisation of the TfR, acid wash and trypsin cleavage techniques were used: with acid wash the extracellular bound Tf was removed, and with trypsin the extracellular TfR was cleaved at its trypsin-sensitive site. Results showed that approximately 70 – 80 % of the associated Tf is acid and trypsin resistant, indicating that most of the receptor-bound Tf is actively internalised within 1 hour.

BBB properties are induced by astrocytic endfeet (3). Therefore, we have investigated the TfR expression and endocytosis in the absence or presence of astrocytic factors in the cell culture medium. The TfR expression and endocytosis were not changed in the absence or presence of astrocyte-conditioned medium. This is consistent with the "no change" in iron and Tf concentrations in astrocyte-conditioned medium compared to normal cell culture medium. This "no change" was expected, since iron concentrations are the main factor regulating the TfR expression.

For the validation of the TfR for drug targeting, horseradish peroxidase (HRP, 40 kDa) was conjugated to a primary amine group in Tf, in a 1:1 ratio (**chapter 4**). HRP was chosen as a model drug, since it is a well known marker for BBB research (11). The association of the Tf-HRP conjugate by BCEC *in vitro*, was linear up to 10 μg/ml. The time to reach equilibrium at 37 °C was 1 hour, which is similar to the results we obtained with ^{125}I-Tf. Furthermore, with the iron scavenger DFO the association of the Tf-HRP conjugate was increased 2.5 fold.

More importantly, results have shown that both ^{125}I-Tf, as well as Tf-HRP are endocytosed via clathrin-coated vesicles, associated with receptor mediated endocytosis (12, 13). This indicates that the conjugation of a 40 kDa protein drug to a primary amine of Tf, does not modify the mechanism of receptor-ligand internalisation. Therefore, we concluded that the TfR is suitable for drug targeting and delivery to the brain.

3. Liposomes tagged with transferrin for drug delivery to the blood-brain barrier

We have extended our research on drug delivery to the brain from Tf-HRP conjugates to Tf-tagged liposomes. By using a liposomal drug carrier the ratio of the number of drug molecules per targeting vector (i.e. Tf) can be improved. In addition, it is not necessary to chemically modify the drug. Finally, for *in vivo* applications the drug is

protected against degradation in serum. Liposomes themselves are biodegradable, have an acceptable safety profile and their circulation time *in vivo* can be improved by sterically stabilising the liposomes with polyethylene glycol (PEG). Zhang *et al* (2003) have shown that weekly injections of liposomes containing PEG and a protein (monoclonal antibody) did not result in toxicity in rats after 6 weeks.

To be able to couple Tf to the PEG end of the liposomes, PEG with a maleimide group at the distal end of the molecule has been used. Tf is modified at a primary amine group with SATA to insert a thiol group for the irreversible coupling to maleimide. During the preparation of the liposomes it is important to have a high ligand coupling efficiency, as well as to maintain the receptor recognition properties of Tf. For the coupling efficiency the stability of both the thiol group and the maleimide group is important, while for receptor recognition it is important that Tf retains its two iron atoms. Holo-Tf (containing 2 iron atoms) has at least a 1000-fold higher affinity for the TfR than mono-ferric Tf or apo-Tf. In general, EDTA is used for the protection of thiol groups, but EDTA is also a potent metal scavenger. We have optimised the preparation of Tf-tagged liposomes by using TCEP, a thiol protectant which does not scavenge iron from Tf (**chapter 5**).

To determine the association of liposomes by BCEC *in vitro*, HRP was chosen as a liposomal marker (**chapter 6**). HRP is an enzyme, which does not cross the BBB by itself (11); its activity can be readily quantified. Therefore, the activity of the protein drug, rather than the amount of associated drug can be determined. Our data suggest that Tf-tagged liposomes bind to the TfR and are subsequently internalised and routed towards the lysosomes, where HRP is degraded. As a result, we find higher values for the binding (at 4 °C) compared to the association (at 37 °C) of Tf-tagged liposomes. Both the binding and association of Tf-tagged liposomes is consistently higher than of non-tagged liposomes. This indicates that tagging liposomes with Tf does have a beneficial effect for the targeting of liposomes to BCEC *in vitro*. The additional advantage of targeting Tf-tagged liposomes to the TfR is the circumvention of the efflux transporter P-glycoprotein (14, 15). This enables the delivery of drugs to the brain which are substrates for P-glycoprotein, for example anti-tumour drugs or anti-viral drugs.

The Tf-HRP conjugates (**chapter 4**) and the Tf-tagged liposomes (**chapter 6**) are differently endocytosed by BCEC *in vitro*. While our data confirm that Tf-HRP conjugates are endocytosed via clathrin-coated vesicles, the mechanism of endocytosis of

Tf-tagged liposomes is yet unclear. Also, there is a difference in intracellular fate of Tf-HRP conjugates and Tf-tagged liposomes. From literature it is known that non-targeted liposomes are directed towards lysosomes. However, our Tf-tagged liposomes were also directed to the lysosomes. This was not expected since the TfR-Tf complex, is directed towards endosomes upon internalisation. Tf-HRP conjugates, either stay in the endosomes or are released from the endosomes into the cytosol. A fraction of the Tf-HRP conjugates might also be directed to the lysosomes. However, we assume that this is only a small fraction, since the association of Tf-HRP is higher than the binding. The difference in association and intracellular routing between the Tf-HRP conjugates (cytoplasm) and the Tf-tagged liposomes (lysosomes) can be explained by their size (figure 1). Tf has a Stokes-Einstein ratio of approximately 4 nm (16), and HRP of approximately 3 nm (17). In contrast, a liposome is 100 nm in diameter.

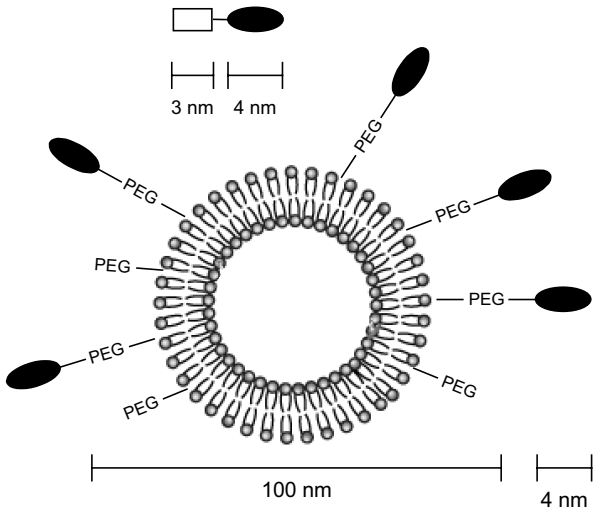

Figure 1: Schematic comparison between HRP encapsulated in a Tf-tagged liposome and a Tf-HRP conjugate. Tf (black oval) is approximately 4 nm (16). HRP (square) has a similar size (approximately 3 nm (17)), while a liposome is 100 nm in diameter.

The difference in size might be the main determinant of the intracellular routing of a particle conjugated to Tf. This information can be used to select either a Tf-drug conjugate or a Tf-tagged liposome, depending on the intracellular target. For example, for the treatment of lysosomal storage disease, it can be useful to target drugs to the

lysosomes, while for other diseases, such as inflammatory disease conditions the cytosol or the nucleus would be more appropriate as a target (18).

3.1 Liposomal drug delivery under inflammatory conditions

Inflammatory disease conditions, such as bacterial meningitis, alter the BBB functioning (19). Lipopolysaccharide (LPS) from gram negative bacteria plays a major role in this altered BBB functioning. Therefore, we have evaluated the TfR expression level, as well as the association after pre-incubation of BCEC with LPS (**chapter 3**). The TfR expression level at 4 °C was not changed after 2 hours pre-incubation with LPS, nor after overnight pre-incubation. However, the cellular protein level after 2 hours incubation with LPS was increased significantly, which gave a biased result when the TfR expression level was expressed per mg cell protein. Since it is known that LPS induces the synthesis of so-called acute phase proteins (20), we have corrected our data for cellular protein levels of the control situation. The association of Tf by the TfR was also not changed after pre-incubation with LPS. These results indicate that the TfR is also suitable for drug targeting and delivery under inflammatory disease conditions.

Targeting liposomes under inflammatory disease conditions was assessed by loading the liposomes with N-acetyl-L-cysteine (NAC). NAC is a hydrophilic free radical scavenger, which can prevent LPS-induced opening of the tight junctions of the BBB *in vitro* (19). In this study, BCEC were co-cultured with rat astrocytes, to form a tight monolayer as measured by TransEndothelial Electrical Resistance (TEER). Overnight pre-incubation with Tf-tagged liposomes loaded with NAC protected the BBB *in vitro* against LPS-induced opening of the tight junctions. However, liposomes that contained just buffer had the same protective effect. Pre-incubation of liposomes with LPS showed a protective effect after at least 6 hours of pre-incubation: the decrease in TEER was less pronounced compared to LPS that was not pre-incubated with liposomes. In addition, pre-incubation of liposomes with fluorescently labelled LPS showed a time-dependent decrease of 15 – 20 % in "free" LPS concentration. In conclusion, it was shown that liposomes have the ability to "scavenge" LPS, thereby decreasing the "free" LPS concentration and thus the LPS effect (**chapter 7**). These results indicate that liposomes are not only suitable as a drug carrier, but also have an intrinsic effect under

inflammatory disease conditions. It needs to be investigated whether the association of LPS by liposomes influences the internalisation of the liposomes.

In addition, NAC might not be the most suitable anti-inflammatory drug, since it is very hydrophilic and can only scavenge one radical per drug molecule. Therefore, more potent scavenger should be considered. Superoxide dismutase (SOD), for example, is an enzyme which can scavenge multiple radicals. Corvo *et al* (2002) have successfully applied liposomes carrying SOD for the treatment of rheumatoid arthritis, a chronic inflammation in joints (21). As an alternative approach, Muzykantov *et al* (1996) have targeted SOD-conjugates to pulmonary endothelium (22). So far, we have used HRP to evaluate drug delivery to the BBB *in vitro*. HRP is not only cheaper, but is also less sensitive to loss of activity. However, the results we have obtained with Tf-HRP conjugates and Tf-tagged liposomes can be extrapolated to SOD. SOD should be delivered to the cytosol, where it can exerts its action. Therefore, Tf-SOD conjugates would be favourable for drug delivery, since these are not primarily directed to the lysosomes. Alternatively, liposomes with endosomal escape possibilities (e.g. with pH dependent fusogenic peptides) can be considered for drug delivery. These liposomes will be discussed in section 4.2.

4. Future perspectives for drug delivery to the brain

The research described in this thesis has been focussing on the use of the TfR for drug delivery to the brain. For this, the endogenous ligand was used, which shows a high affinity for the TfR. The Tf concentration in serum is high, limiting the *in vivo* applicability as a targeting vector. In addition, the TfR is not only expressed at the BBB, but also on hepatocytes, erythrocytes, monocytes and intestinal cells (23). The TfR is therefore not very selective for drug delivery to the brain. However, extensive research by Pardridge *et al* has shown that drug delivery via the TfR is effective (1, 29, 30). Our research was performed *in vitro*, to be able to characterise the TfR and its potential use for drug targeting more mechanistically. It is known that the ideal *in vitro* BBB model to determine endocytosis and transcytosis of compounds and to extrapolate results to the *in vivo* situation does not exist. However, we have used primary cultured bovine BCEC, which are cultured in the presence of astrocyte-conditioned medium. These BCEC are

well characterised for their BBB-like properties, such as the expression of P-glycoprotein, cadherin 5, P-selectin and α_V-integrin, and the activity of γ-GTP enzyme (4). In addition, we have fully characterised the TfR on these BCEC (**chapter 3**). The limitations of an *in vitro* BBB model often involve the paracellular pathway. However, we have focussed on the very first step of drug delivery to the brain: the binding of Tf, Tf-HRP conjugates or Tf-tagged liposomes to the TfR and the subsequent internalisation by the BCEC. Therefore, the primary cultured BCEC were a good model to study the TfR.

4.1. Targeting vectors for brain drug delivery via the transferrin receptor

Our *in vitro* research suggests that the TfR is suitable for drug delivery to the brain. This is in agreement with the *in vivo* research performed by Pardridge and co-workers, who used the monoclonal antibody OX-26 against the rat TfR as a targeting vector. Both Tf and OX-26 have their advantages and disadvantages as a targeting vector. Tf is the endogenous ligand for the TfR, with a relatively high concentration in plasma (22 – 35 μM (23)). Therefore, when using Tf as a targeting vector, competition for the receptor-binding site can be expected. However, our research with Tf-tagged liposomes has shown that liposomes with multiple Tf molecules on their surface, bind to the TfR on BCEC *in vitro* and are internalised in the presence of serum (**chapter 6**). In contrast, OX-26 does not bind to the Tf-binding site and is therefore not displaced by endogenous Tf. However, OX-26 is an antibody against the rat TfR and does not fit into the human TfR. Moreover, rat antibodies will cause immunogenic reactions in humans, unless they are humanised. The preparation of humanised or chimeric antibodies is difficult. In some cases this may lead to a loss of affinity for the target receptor (24).

The TfR is responsible for iron transport to the brain. So far, the intracellular trafficking of Tf and OX-26 upon internalisation via the TfR has not yet been elucidated. Some literature reports suggest transcytosis of Tf across the BCEC, while others claim endocytosis of Tf, followed by an intracellular release of iron and a subsequent return of apo-Tf to the apical side of the BCEC (12, 25, 26). Moos and Morgan (2000) have shown that the transcytosis of iron exceeds the transcytosis of Tf across the BBB, supporting the second theory (27). Furthermore, these authors have

proposed a new theory in which the TfR-Tf complex is transcytosed to the basolateral side of the BCEC, where Tf remains bound to the TfR, but iron is released into the brain extracellular fluid (28). Subsequently apo-Tf bound to the TfR will recycle back to the apical side of the BBB. This theory is supported by data from Zhang and Pardridge (2001) who found a 3.5 fold faster efflux from brain to blood of apo-Tf than holo-Tf (25).

The mechanism of transcytosis of OX-26 is not yet fully elucidated either. Pardridge *et al* have shown efficient drug targeting and delivery to the brain *in vivo* by applying OX-26 (examples can be found in (26, 29-31)). In contrast, Broadwell *et al* (1996) have shown that both Tf and OX-26 are able to cross the BBB, but that the transcytosis of Tf is more efficient (32). Furthermore, Moos and Morgan (2001) have shown that OX-26 mainly accumulates in the BCEC and not in the post-capillary compartment (33). In addition, iron deficiency did not increase OX-26 uptake in rats. Our data as well as literature reports show that iron deficiency causes an increase in TfR expression (**chapter 3**, (6, 34)). Therefore, it is expected that the uptake of OX-26 would increase as well. These data suggest that OX-26 transcytosis might result from a high-affinity accumulation by the BCEC, followed by a non-specific exocytosis at the basolateral side of the BCEC (33). In addition, these authors found a periventricular localisation of OX-26 which suggest that OX-26 probably also is transported across the blood-CSF barrier. Although the mechanism of transcytosis of Tf and OX-26 may not yet be fully elucidated, it is important to realise that drug delivery to the brain via the TfR is efficient.

Preferably, a targeting vector directed to the TfR would be small, non immunogenic and should initialise internalisation of the TfR upon binding. Xu *et al* (2001) 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 (35). The molecular weight of this antibody fragment, including the lipid anchor was approximately 30 kDa. In addition, Lee *et al* (2001) have used a phage-display technique to find small peptide ligands for the human TfR. They obtained a 7- and a 12-mer peptide, that bind to a different binding site than Tf and are internalised by the TfR. Although these small peptides can also exert immunogenic reactions in humans, they are promising ligands for drug targeting to the TfR on the BBB.

In addition to antibody fragments and peptides, small synthetic ligands can offer great promise for drug delivery to the TfR. These synthetic ligands can either be

developed by molecular modelling of TfR binding sites or by screening of large compound banks. Preferably, a small synthetic ligand should not bind to the Tf-binding site, to avoid competition from endogenous Tf.

4.2 Drug conjugates versus liposomal drug carriers

The research described in this thesis has been focused on the use of Tf-drug conjugates and Tf-tagged liposomes. Drug conjugates are much smaller than liposomes: Tf-HRP conjugates are about 14 times smaller, based on the Stokes-Einstein radii of Tf and HRP (approximately 4 and 3 nm, respectively), than Tf-tagged liposomes (approximately 100 nm in diameter). It is likely that the internalisation of smaller particles by the BCEC is easier and thus quicker. Clathrin-mediated endocytosis, which is associated with the TfR, allows for particles up to 120 nm to be internalised (36). Rejman *et al* (2004) have shown that non-targeted particles of 50 and 100 nm in diameter were internalised by clathrin-mediated endocytosis, and with similar kinetics (37). Particles which were 200 nm in diameter were also internalised via clathrin-mediated endocytosis, but their uptake was slower. The amount of 100 and 200 nm particles that were internalised was respectively 3 – 4 and 8 – 10 times lower compared to the uptake of 50 nm particles (37). Although the uptake of larger particles is lower, these particles can encapsulate more drug molecules, resulting in a similar intracellular drug concentration.

Besides internalisation, intracellular trafficking also seems to be different for Tf-drug conjugates and Tf-tagged liposomes. Tf-tagged liposomes are directed towards the lysosomes, while Tf-HRP conjugates stay in the endosomes or are released into the cytosol. Beyer *et al* (2001) have found similar results for doxorubicin: liposomal doxorubicin showed less endocytosis than free doxorubicin, but the intracellular distribution of liposomal doxorubicin was similar to free doxorubicin (38). In contrast, Tf- and BSA-doxorubicin conjugates were differently distributed. These findings are similar to our results and could imply that depending on the intracellular target, a Tf-drug conjugate or a Tf-tagged liposome is preferred.

The Tf-tagged liposomes we have used were made of conventional lipids (egg phosphatidylcholine and cholesterol) and were sterically stabilised with PEG. Over the

years new techniques have been developed to prevent the liposomal drug from degradation by the lysosomes. First, pH sensitive liposomes have been developed, which dissociate at low pH, releasing their contents in the endosomes. Secondly, in combination with a fusogenic peptide, such as GALA or TAT (39, 40), the endosomal membrane is disrupted and the drug is released into the cytosol. Recently, Kakudo *et al* (2004) have demonstrated that Tf-modified liposomes equipped with a pH sensitive fusogenic peptide efficiently fuse with the endosomal membrane (41). This drug delivery system allows for the efficient delivery of gene therapeutics to the brain.

It depends on the characteristics of the drug, as well as on the intracellular target whether a drug conjugate or a liposomal drug carrier is the best option for the delivery of the drug to the target site. If the drug is a hydrophilic protein which is rather large, the encapsulation efficiency by a small liposome is not as high as it is for smaller drug molecules. On the other hand, for the incorporation of a drug into a liposome it is not necessary to chemically modify the drug. Furthermore, the drug is protected against degradation in serum. For gene therapeutics, stability in serum is an important issue, since nucleic acids are susceptible to ribonuclease degradation. Therefore, liposomal drug carriers are more suitable for gene therapeutics compared to drug conjugates. Recently, interference RNA (RNAi) has shown great promise for silencing gene expression at the post-transcriptional level (42, 43). Zhang *et al* (2004) used RNAi to silence oncogenic genes in solid cancers, by using liposomes tagged with antibodies against the TfR, as well as against the insulin receptor (44).

In summary, it depends on the drug characteristics, as well as on the intracellular target, whether a drug conjugate or a liposomal drug carrier is preferred for the selective delivery to the BBB and the brain.

4.3. Brain drug delivery: a selection of targets

The TfR is suitable for drug targeting to the brain, since it is abundantly expressed at the BBB and is actively endocytosing its ligand. However, by long-term use of the TfR for drug targeting the uptake of Tf might be disturbed and this may affect the intracellular iron homeostasis. Iron plays a role in metabolic processes and acts as co-factor for several enzymes. Long-term iron deficiency in the brain of children has been

shown to lead to impaired cognition and abnormal behaviour (23). In contrast, an excess of iron may lead to the formation of free radicals and tissue damage (23). This may be a contributing factor in the development of several neurological diseases (Baker & Morgan 1994), such as Alzheimer's or Parkinson's disease. However, the BBB is a dynamic organ, which tightly regulates the brain homeostasis. Furthermore, the large intracellular pool of TfR (90 % of the total TfR expression, **chapter 3**) and the efficient transcriptional control of the TfR, as well as ferritin (7, 8) suggest that iron sequestration by cells or organs is strictly regulated. Therefore, the iron homeostasis might not be influenced by targeting the TfR, but it may cause a receptor downregulation and thus a lower targeting efficiency.

Other physiological drug targeting and delivery strategies that are under investigation involve adsorptive-mediated transcytosis, carrier-mediated transcytosis or receptor-mediated transcytosis. Zhang *et al* (2004) have used liposomes tagged with two antibodies: one against the TfR, and the other against the insulin receptor (44, 45). This double labelling enables the liposomes to cross the BBB as well as the cell- and the nuclear membrane.

Recently, new techniques, such as gene array technologies and subtractive antibody expression cloning have been developed which may lead to new targets at the BBB (46-49). Shusta *et al* have shown a high and specific expression of the Lutheran membrane glycoprotein and the membrane cofactor protein CD46 in BBB endothelial cells compared to other organs, such as kidney and liver (50, 51). These techniques could also be used to find targets for drug delivery which are up-regulated in a disease state (e.g. under inflammatory disease conditions).

4.4 Learning from pathogens for brain drug delivery

Crossing the BBB via endogenous, physiological transport systems might not be the only attractive strategy for drug delivery to the brain. Viral, bacterial, fungal and parasitic pathogens are able to cross the BBB and infect the brain. The limited number of pathogens crossing the BBB suggests that these have very specific attributes (52). Understanding the mechanisms by which these pathogens cross the BBB might not only help to develop new treatments against infections, but can also help to develop new

targeting strategies for drug delivery to the brain for the treatment of other diseases. Bacteria, which replicate intracellularly are likely to use infected leukocytes as a 'Trojan horse' to cross the BBB, while it is yet unknown how extracellularly replicating bacteria cross the BBB (52).

Another example of a pathogen crossing the BBB, is a parasite, *Toxoplasma gondii*, which can not only cross the intestinal barrier, but also the placental barrier, the blood-retina barrier and the BBB (53). It is yet unknown how this parasite is able to cross these barriers, although some evidence suggests that there are analogies to leukocyte trafficking. Leukocyte trafficking across the BBB is caused by a cascade of effects. The first step is the recognition of adhesion molecules on the surface of leukocytes by their corresponding ligands on the BCEC, after which they are to cross the BBB paracellularly (54). Parasites could use a similar mechanism of paracellular transport, after recognition and possible activation of a cellular marker.

Pathogens can also bypass the BBB to reach the brain (52, 55). This can be achieved by using retrograde axonal transport through sensory or motor fibers. Prion disease is an example of a pathogen that bypasses the BBB in this way. Recently, Haik *et al* (2004) have reported that prions probably use the sympathetic noradrenergic neurons to reach the brain (55).

5. Conclusion

The research described in this thesis was initiated to meet the challenge of drug delivery across the BBB. The focus of this thesis was to explore the possibilities for drug delivery to the brain via the TfR. Although drug delivery to the brain via the TfR is not selective, it is effective. We have chosen for a mechanistic approach in which the binding and association of Tf, Tf-drug conjugates and Tf-tagged liposomes by BCEC *in vitro* was determined. The efficiency of delivery depends mainly on the size of the cargo, a 40 kDa protein does not affect the association of Tf by the TfR, while a liposomal drug carrier (100 nm in diameter) affects both the association and the intracellular routing.

In conclusion, results obtained in our *in vitro* BBB model have shown that the TfR is an attractive target for the delivery of drugs to the brain.

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