

## Targeting the transferrin receptor for brain drug delivery

Bendix Johnsen, Kasper; Burkhart, Annette; Bohn Thomsen, Louiza; Andresen, Thomas Lars; Moos, Torben

*Published in:*  
Progress in Neurobiology

*DOI (link to publication from Publisher):*  
[10.1016/j.pneurobio.2019.101665](https://doi.org/10.1016/j.pneurobio.2019.101665)

*Creative Commons License*  
CC BY-NC-ND 4.0

*Publication date:*  
2019

*Document Version*  
Accepted author manuscript, peer reviewed version

[Link to publication from Aalborg University](#)

*Citation for published version (APA):*  
Bendix Johnsen, K., Burkhart, A., Bohn Thomsen, L., Andresen, T. L., & Moos, T. (2019). Targeting the transferrin receptor for brain drug delivery. *Progress in Neurobiology*, 181, Article 101665. <https://doi.org/10.1016/j.pneurobio.2019.101665>

### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal -

### Take down policy

If you believe that this document breaches copyright please contact us at [vbn@aub.aau.dk](mailto:vbn@aub.aau.dk) providing details, and we will remove access to the work immediately and investigate your claim.

## Accepted Manuscript

Title: Targeting the transferrin receptor for brain drug delivery

Authors: Kasper Bendix Johnsen, Annette Burkhart, Louiza Bohn Thomsen, Thomas Lars Andresen, Torben Moos

PII: S0301-0082(19)30134-0  
DOI: <https://doi.org/10.1016/j.pneurobio.2019.101665>  
Article Number: 101665

Reference: PRONEU 101665

To appear in: *Progress in Neurobiology*

Received date: 14 May 2019  
Revised date: 10 July 2019  
Accepted date: 18 July 2019

Please cite this article as: Bendix Johnsen K, Burkhart A, Bohn Thomsen L, Andresen TL, Moos T, Targeting the transferrin receptor for brain drug delivery, *Progress in Neurobiology* (2019), <https://doi.org/10.1016/j.pneurobio.2019.101665>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Targeting the transferrin receptor for brain drug delivery

Kasper Bendix Johnsen<sup>1\*</sup>, Annette Burkhart<sup>2</sup>, Louiza Bohn Thomsen<sup>2</sup>, Thomas Lars Andresen<sup>1</sup> & Torben Moos<sup>2</sup>

<sup>1</sup>Center for Nanomedicine and Theranostics, Department of Health Technology, Technical University of Denmark, Denmark. <sup>2</sup>Laboratory for Neurobiology, Biomedicine, Institute of Health Science and Technology, Aalborg University, Aalborg, Denmark.

\* Correspondence:

Kasper Bendix Johnsen, PhD  
Center for Nanomedicine and Theranostics  
Department of Health Technology  
Technical University of Denmark  
Produktionstorvet, Building 423  
2800 Kgs. Lyngby, Denmark  
E-mail: kasjoh@dtu.dk  
Phone number: 0045 4525 8137

### Highlights

- Targeting of the transferrin receptor is one of the most efficient ways of obtaining brain drug delivery.
- Despite decades of development, very little clinical progression has been made, although few clinical trials are beginning to emerge.
- New innovations in terms of modifications of the antibody binding mode has yielded impressive leaps forward in our understanding of the transport process.
- Many parameters in the transferrin receptor-mediated transport across the blood-brain barrier are still poorly understood.
- Increased understanding of factors like intracellular sorting and subsequent long-term brain retention of TfR-targeted drug delivery systems is needed to fulfill the ambition of clinical use.

### Abstract

Obtaining efficient drug delivery to the brain remains the biggest challenge for the development of therapeutics to treat diseases of the central nervous system. The main obstacle is the blood-brain barrier (BBB), which impedes the entrance of most molecules present in the systemic circulation, especially large molecule drugs

and nanomedicines. To overcome this obstacle, targeting strategies binding to nutrient receptors present at the luminal membrane of the BBB are frequently employed. Amongst the numerous potential targets at the BBB, the transferrin receptor (TfR) remains the most common target used to ensure sufficient drug delivery to the brain. In this review, we provide a full account on the use of the TfR as a target for brain drug delivery by describing the function of the TfR in the BBB, the historical background of its use in drug delivery, and the most recent evidence suggesting TfR-targeted medicines to be efficient for brain drug delivery with a clear clinical potential.

### **Statement of significance**

Targeting of the TfR remains the most important tool to improve brain drug delivery. The recent years has seen both technological advancements as well as introduction of the strategy to clinical trials. This review provides the first full account of the field of TfR-targeted brain drug delivery systems to be published, including a description of the relevance of TfR-targeting in relation to antibody-based medicines and large nanocarriers used to treat brain diseases.

### **Abbreviations**

AAV – Adeno-associated virus

ADCC – Antibody-dependent cellular cytotoxicity

AJ – Adherens junction

BBB – Blood-brain barrier

BCEC – Brain capillary endothelial cell

BCRP – Breast cancer resistance protein

BDNF – Brain-derived neurotrophic factor

CNS – Central nervous system

CED – Convection-enhanced delivery

CSF – Cerebrospinal fluid

Da – Dalton

DAB – Diaminobutyric polypropylenimine dendrimer

Dcytb – Duodenal cytochrome b

DMT – Divalent metal transporter

EAE – Experimental autoimmune encephalitis

EGF – Epidermal growth factor

EPO – Erythropoietin

Fab – Fragment antigen binding

Fc – Fragment crystallizable

FGF – Fibroblast growth factor

GDNF – Glial-derived neurotrophic factor  
GLUT – Glucose transporter  
GPI – Glycosylphosphatidylinositol  
ICAM – Intercellular adhesion molecule  
%ID – Fraction of injected dose  
IDS – Iduronate-2-sulfatase  
IDUA –  $\alpha$ -L-iduronidase  
JAM – Junctional adhesion molecule  
LAT – Large amino acid transporter  
LRP – Low-density lipoprotein receptor related protein  
MPS – Mucopolysaccharidosis  
NTBI – Non-transferrin-bound iron  
NVU – Neurovascular unit  
OATP – Organic anion-transporting peptide  
P-gp – P-glycoprotein  
PANAM – Polyamidoamine  
PbMLA – Poly( $\beta$ -L-malic acid)  
PCL – Polycaprolactone  
PEG – Polyethylene glycol  
PET – Positron emission tomography  
PLA – Poly-lactic acid  
PLGA – Poly(lactic-co-glycolic acid)  
PNA – Peptide nucleic acids  
PPE – Polyphosphoester  
scFv – Single-chain variable fragment  
SGSH – N-sulfoglucosamine sulfohydrolase  
Steap – Six-transmembrane epithelial antigen of the prostate  
Tf – Transferrin  
TfR – Transferrin receptor  
TJ – Tight junction  
TNFR – Tumor necrosis factor receptor  
TPGS – D-alpha-tocopheryl polyethylene glycol 1000 succinate  
VE – Vascular endothelial  
VEGF – Vascular endothelial growth factor  
VIP – Vasoactive intestinal peptide

ZO – Zonula occludens

## **Table of contents**

Abstract

Statement of significance

1. Introduction

1.1. The blood-brain barrier

1.2. Brain capillary endothelial cells form the blood-brain barrier

1.3. Transport mechanisms at the blood-brain barrier

2. Iron uptake and metabolism in the brain

2.1. Structure and function of Tf and the TfR

2.2. Mechanism of TfR endocytosis and iron uptake into the brain

3. The TfR as a target for brain drug delivery

3.1. Ligands

3.2. Drug conjugates targeting the TfR

3.3. Liposomes

3.4. Polymeric nanoparticles

3.5. Gold nanoparticles

3.6. Miscellaneous nanoparticles

4. Challenges for current TfR drug delivery strategies

4.1. Does the TfR transcytose drugs into the brain?

4.2. TfR-targeted brain drug delivery in disorders with loss of blood-brain barrier integrity

4.3. Challenges in addition to transcytosis of the blood-brain barrier

4.4. Biodistribution properties and potential side effects

5. Modifications to existing TfR drug delivery strategies to improve brain uptake

5.1. Affinity

5.2. Avidity

5.3. pH

5.4. Cell-penetrating peptides

5.5. Degradation or activation of nanoparticles

5.6. Administration routes

6. Conclusion

Acknowledgements

Authorship contributions

References

## Abbreviations

### 1. Introduction

Millions worldwide suffer from neurological disorders, and even more will be affected by e.g. Alzheimer's and Parkinson's disease, and cerebrovascular diseases in the coming decades (Murray et al., 2012; Whiteford et al., 2015). The possibilities for treatment of neurological diseases are hampered because drug delivery to the brain is hindered by the blood-brain barrier (BBB). The BBB functions to regulate the fluxes of molecules between the systemic circulation and the brain (Abbott, 2013). Development of successful strategies to bypass the integrity of the BBB is thus pivotal to obtain tools that will allow for treatment of diseases of the central nervous system (CNS). To develop such bypassing strategies, it is also of great importance to understand the morphological and physiological features that characterize the BBB. A promising strategy is to target CNS-active drugs towards BBB-associated carrier and receptor proteins, among which a targeting approach against the transferrin receptor (TfR) that is expressed by brain capillary endothelial cells (BCECs) is the most widely used (Johnsen and Moos, 2016).

In this review, the current knowledge on the role of the TfR in brain iron uptake is delineated. This is followed by full coverage of the functions of the TfR and its ligands together with a thorough summary of studies investigating the delivery of TfR-targeted antibodies and nanomedicines to the brain parenchyma. The challenges of the TfR-targeting strategy are discussed with emphasis on the possibilities to improve this type of brain drug delivery to become the brain medicines of the future.

#### *1.1. The blood-brain barrier*

The BBB is located within the microvasculature of the brain, and it regulates passage of molecules from the blood to the brain (Burkhart et al., 2014). The BCECs of the brain's microvasculature form the BBB (Abbott, 2013), while pericytes and astrocytes support the BCECs barrier function during embryonic formation and maintenance of the BBB (Figure 1)(Armulik et al., 2010; Daneman et al., 2010b; Sengillo et al., 2012). Pericytes are embedded in the shared basement membrane on the abluminal side of the BCECs (Armulik et al., 2011), and they contribute to the tightness of the BBB by decreasing transcytosis and by inducing polarization of astrocyte end-feet (Armulik et al., 2010; Daneman et al., 2010a). The end-feet of astrocytes cover a substantial part of the abluminal side of the BCECs and the pericytes (Abbott et al., 2006). These perivascular astrocytes play a significant role in intercellular tightening of the BBB and regulation of polarized expression of BCEC transporters (Abbott et al., 2006; Wolburg et al., 2009).

#### *1.2. Brain capillary endothelial cells form the blood-brain barrier*

The BCECs differ from the peripheral endothelial cells by having less endocytic vesicles, specialized transcellular transport systems, very low paracellular passage, and by lacking fenestrations (Abbott et al.,

2010). The BCECs also express efflux transporters e.g. P-glycoprotein (P-gp) and breast cancer related protein (BCRP), which reduce the entry of amphiphilic drugs by carrier-mediated efflux of some of these drugs from the BCECs to the brain (Abbott et al., 2010). Altogether, the BCECs form a very tight barrier, which is mainly due to a restricted paracellular and transcellular passage (Burkhart et al., 2014).

In the junctions between the BCECs, three groups of junctional proteins are expressed. The first group is the tight junctions (TJs), which are the limiting factor for the paracellular passage since they only enable passage of very small hydrophilic molecules (Haseloff et al., 2015)(Figure 1). The TJ proteins include claudin 1, 3 and 5, which are connected to the BCECs cytoskeleton via the membrane-associated zonula occludens proteins 1-3 (ZO 1-3)(Nitta et al., 2003; Wolburg et al., 2003). Furthermore, in addition to the TJs are the tight junction-associated marvel proteins, occludin and tricellulin (Hirase et al., 1997; Mariano et al., 2013). The second group is the adherence junction (AJs) proteins, which include vascular endothelial cadherin (VE-cadherin), catenins, and platelet endothelial cell adhesion molecules (PECAM)(Wolburg and Lippoldt, 2002). The AJs are important for interconnection of the BCECs, hence giving structural tissue support (Abbott et al., 2010). The third group is denoted by the junctional adhesion molecules (JAMs)(Abbott et al., 2010). The function of the JAMs is not fully uncovered, but they seem to have a role in cell adhesion of leukocytes (Burkhart et al., 2014). The adherence junctions, ZO 1-3 and JAMs does not directly take part in limiting the paracellular passage but support the structure in forming a tight barrier (Haseloff et al., 2015). The limited paracellular passage at the BBB is mainly obtained by the presence of claudins and occludins, with claudin 5 being the most important protein for maintaining the tight BBB (Haseloff et al., 2015).

### *1.3. Transport mechanisms at the blood-brain barrier*

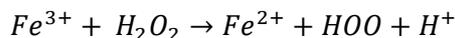
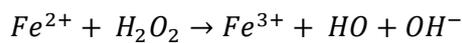
The transcellular passage through the BCECs (Figure 2) can take place either via 1) cell entry by leukocytes; 2) carrier-mediated influx of e.g. glucose by glucose transporter 1 (GLUT-1), amino acids by e.g. the L-type amino acid transporter 1 (LAT-1) and small peptides by e.g. organic anion-transporting peptide-B (OATP-B); 3) paracellular passage of small hydrophobic molecules; 4) adsorption-mediated transcytosis of e.g. albumin and cationized molecules; 5) passive diffusion of lipid soluble, non-polar solutes, including CO<sub>2</sub> and O<sub>2</sub>; and 5) receptor-mediated transcytosis of e.g. insulin by the insulin receptor and Tf by the TfR (Abbott, 2013; Abbott et al., 2006; Begley and Brightman, 2003). These transport systems have all been explored for their use as targets for drug delivery to the brain (Abbott et al., 2010; Burkhart et al., 2014; Lichota et al., 2010). Among these, the receptor-mediated transport route is the focus of this review. The purpose of targeting the BBB receptors is to use ligands or monoclonal antibodies as ferrying molecules, thereby exploiting the ability of these molecules to bind to receptors and induce transport of the drug into the brain. To secure exclusive uptake in the brain following intravascular administration, a drug-bearing ligand needs its target receptor to be either upregulated at the BBB or limited to be expressed by the BCECs.

A plethora of studies have explored the use of BBB receptors and their ligands for developing strategies to bypass the BBB (see e.g. Table 4 in *Abbott et al. (2010)*(Abbott et al., 2010)). The TfR is of high interest due to its exclusive expression in the BCECs compared to other types of endothelial cells in the periphery (Jefferies et al., 1984). Although the theory of using BCECs receptors for drug delivery is promising, many obstacles have been encountered in the process: 1) The endogenous receptor ligand can in some cases outcompete binding of the drug-bearing ligand (Lichota et al., 2010), 2) The affinity of the drug-bearing ligand to the receptor can also influence the uptake and thereby form either too weak or too strong bindings to the receptors to undergo transcellular transport (Burkhart et al., 2014; Yu et al., 2011), 3) The drug-bearing ligand can be a substrate for the efflux transporter and be transported out of the BCECs after being internalized in the cell (Begley, 2004), 4) The endothelial glycocalyx denote an obstacle for the drug bearing ligand by filtering molecules before they reach the BCECs luminal cell membrane (Brightman and Kaya, 2000), and 5) The BCECs basement membrane can hinder transport from the BCECs and into the brain parenchyma (Muldoon et al., 1999). These obstacles need to be overcome for a brain drug delivery strategy to become successful, and amongst the different strategies that are known and have been studied, targeting TfRs at the BBB represents one of the most relevant and efficient ones (in addition to e.g. the insulin receptor (Giugliani et al., 2018; Pardridge et al., 2018a)).

## 2. Iron uptake and metabolism in the brain

To evaluate the potential of TfR as a target for brain drug delivery a detailed understanding of the process of brain iron uptake via the TfR is necessary. Such a detailed understanding will aid in the interpretation of the evidence suggesting TfR-mediated brain drug delivery to be either possible or impossible. Importantly, there is still no universal consensus as to the sorting of TfRs in BCECs, which can result in seemingly similar brain drug delivery studies being interpreted in opposite directions. In the next sections, we review the current evidence related to the uptake of iron into the brain parenchyma via TfR-mediated transport through the BCECs.

Iron is an essential molecule for most living organisms since it is an essential component of or a co-factor for a vast number of proteins, including many important enzymes (Beard et al., 1996). Iron is involved in basic cellular processes like oxygen transport, enzyme reactions, oxidation and reduction reactions, and in cell division. Iron additionally functions as a co-factor in the electron transport chain and during DNA synthesis (Duck and Connor, 2016). Although iron denotes an essential element in the cell, it has chemical properties that makes it toxic when present in excess (Anderson and Vulpe, 2009; Duck and Connor, 2016; Moos and Morgan, 2000). In aqueous solutions, iron exists in two forms: the reduced state ( $\text{Fe}^{2+}$ ), referred to as ferrous iron or its oxidative state ( $\text{Fe}^{3+}$ ), referred to as ferric iron. Iron can easily change between its ferrous and ferric form and this transition is the basis of many biological redox reactions, also known as Fenton reactions (Duck and Connor, 2016; Wardman and Candeias, 1996).



When ferrous iron participates in the Fenton reaction by reacting with hydrogen peroxide, it results in the formation of a hydroxyl radical and a hydroperoxyl radical. Both are toxic free radicals and reactive oxygen species with the potential to damage lipids, proteins and DNA, ultimately leading to cell death, especially when present in excess (Anderson and Vulpe, 2009; Chevion, 1988; Duck and Connor, 2016; Stohs and Bagchi, 1995). Extracellularly, iron is bound to the iron-transport protein, Tf, which carry iron between cells in its oxidized, non-toxic form (Moos and Morgan, 2000). Intracellularly, iron remains in its reduced state, and if it is not required immediately for metabolic functions, it gets oxidized and incorporated by the iron storage protein, ferritin. This blocks ferrous iron from participating in unwanted redox reactions, thereby preventing the production of toxic radicals (Anderson and Vulpe, 2009; Skjørringe et al., 2015). Ferritin is comprised of 24 polypeptide subunits and highly conserved among higher eukaryotes (Montemiglio et al., 2019). Two isoforms of the subunits exist, the heavy (H) and the light (L) subunit, and the composition of ferritin seems to be tissue specific. The H subunit is a 21 kDa protein predominantly expressed in the heart, while the L isoform is a 19 kDa protein predominantly expressed in the liver. By transforming ferrous iron to ferric iron, ferritin also exerts oxidative activity making it able to store iron in its non-toxic, ferric form (Beard et al., 1996; Theil, 1987).

### 2.1. Structure and function of Tf and the TfR

In physiological conditions, essentially all iron circulating in plasma is bound to Tf, which is a 78 kDa monomeric glycoprotein and a member of a closely related family of glycoproteins that also includes lactoferrin, melanoTf and ovoTf (Huebers and Finch, 1987; Moos and Morgan, 2000; Morgan, 1979). Tf is one of the most abundant plasma proteins present in concentrations ranging from 25 – 50  $\mu$ M (2 – 4  $\mu$ g/ml) in the normal human adult blood plasma (Anderson and Vulpe, 2009; Luck and Mason, 2012; H. Sun et al., 1999). It is synthesized primarily in the liver (Moos and Morgan, 2000), but locally it is also synthesized in the CNS in the oligodendrocytes and choroid plexus epithelial cells (Aldred et al., 1987; Bloch et al., 1985; Dickson et al., 1985; Dziegielewska et al., 1984), and in the Sertoli cells of the testes (Moos and Morgan, 2000; Skinner et al., 1984; Sylvester and Griswold, 1984). Due to the high abundance of Tf in plasma, only approximately 30 % of the Tf molecules are saturated with iron. Therefore, Tf functions as a buffer molecule if the plasma concentration of iron rapidly increases, hereby preventing the risks of accumulation of toxic non-Tf bound iron (NTBI), which can be severely toxic in the long run as seen in hemochromatosis (Anderson and Vulpe, 2009; Luck and Mason, 2012). Noteworthy, the BBB does not present with excess accumulation of

iron in hemochromatosis, indicating that the brain stands out as the only organ that remains unaffected with high circulating levels of non-Tf-bound iron (Moos et al., 2000).

The Tf molecule is composed of a single polypeptide chain with two domains of equal size, the N and C terminal domains. Each domain contains an iron-binding site, which has an extremely high binding affinity ( $K_D = 10^{-22}$  M) towards a single iron atom (Aisen et al., 1978; Anderson and Vulpe, 2009; Luck and Mason, 2013); hence, each Tf molecule can carry two iron molecules in their ferric form. Since Tf has two binding sites for iron, different forms of Tf exist. Non-iron bound Tf is referred to as apo-Tf, the binding of one iron atom is referred to as mono-ferric Tf, while the binding of two irons is referred to as di-ferric Tf, or simply holo-Tf (Luck and Mason, 2013; 2012; Moos and Morgan, 2000; Tortorella and Karagiannis, 2014). The binding of iron to Tf is pH-dependent. At a physiological pH of 7.4, the binding affinity of Tf to iron is highest, making the binding almost irreversible, but at pH levels lower than 6.5, the binding affinity of Tf to iron is lowered, causing a release of iron (Moos and Morgan, 2000; Morgan, 1983). The primary role of Tf in plasma and interstitial fluids of the body is to function as an iron carrier that accepts iron released from cells and transport it to other cells (Moos and Morgan, 2000; Moos et al., 2007). Due to the hydrophilic nature of the Tf molecule and its high affinity towards the TfR, Tf-bound iron is mainly taken up by cells expressing the TfR (Luck and Mason, 2012; Moos et al., 2007). The Tf molecule undergoes a conformational change when bound to iron, which is important in relation to its selective recognition by the TfR (H. Sun et al., 1999).

Two different TfRs have been recognized. These are referred to as TfR1 and TfR2, of which the TfR1 has been most extensively studied. They show homologies around 45 – 66 % in the extracellular domain but present with different expression patterns in the body. The TfR2 is mainly expressed in tissues involved in regulating iron metabolism, such as the liver and small intestines, while the TfR1 is generally found on the surface of most body cells (Tortorella and Karagiannis, 2014; Trinder and Baker, 2003). Active proliferating cells or cells that have specialized iron requirements, like immature erythroid cells with a large requirement of iron for haemoglobin synthesis, express higher levels of TfR1 (Gatter et al., 1983; Tortorella and Karagiannis, 2014). TfR2 has a 25-fold lower affinity towards Tf compared to TfR1 (Kawabata et al., 2000; Tortorella and Karagiannis, 2014).

The human TfR1 is a transmembrane glycoprotein consisting of two identical subunits of 90 kDa linked by disulphide bonds (the term ‘TfR’ will be used throughout the remainder of this chapter referring to ‘TfR1’). Each subunit is a type II membrane protein made up of a short N-terminal cytoplasmic tail, containing a consensus internalization motif, a hydrophobic transmembrane domain, and a large globular extracellular C-terminal domain. The extracellular domain contains the binding site for Tf. Each subunit can bind one Tf molecule, thereby having the capacity of internalizing four ferric irons during each cycle of TfR-mediated endocytosis (Aisen et al., 1978; Anderson and Vulpe, 2009; Y. Cheng et al., 2004). At physiological pH around 7.4, the TfR binds holo-Tf with the highest affinity ( $K_D \sim 4$  nM). Mono-ferric Tf binds with a lower affinity to the TfR ( $K_D \sim 32 - 36$  nM, depending on which domain of the Tf molecule iron is bound to) than holo-Tf.

Apo-Tf has a very low affinity for the TfR and most likely does not bind at neutral pH (Eckenroth et al., 2011; Luck and Mason, 2012; Mason et al., 2009). However, as the pH decreases the affinity for apo-Tf increases (Luck and Mason, 2013). Approximately 15 – 20 % of the TfRs are residing on the cell surface (Johnsen and Moos, 2016; Luck and Mason, 2013; van Gelder et al., 1995; Visser et al., 2004b). Upon binding of holo-Tf, the Tf-TfR complex is endocytosed through clathrin-coated pits (Mayle et al., 2012; Tortorella and Karagiannis, 2014; Yashunsky et al., 2009).

## 2.2. Mechanism of TfR endocytosis and iron uptake into the brain

Iron must be transported across the BBB to enter the CNS (Skjørringe et al., 2015). The iron uptake is a highly regulated process since the presence of iron in insufficient amounts or in excess within the CNS is associated with neurological diseases (Altamura and Muckenthaler, 2009; Duck and Connor, 2016). The mechanisms of uptake and transport of iron across the BCECs constituting the BBB have been a matter of debate for long (Figure 3)(Benkovic and Connor, 1993; Burdo et al., 1999; 2001; Duck and Connor, 2016; Friden et al., 1991; Lee et al., 2000; Leitner and Connor, 2012; Moos et al., 2007; Moos and Morgan, 2001; Moos et al., 2006; Pardridge et al., 1991; Skjørringe et al., 2015). The endothelial cells of the vasculature do not express TfR despite their immediate access to circulating holo-Tf, except for the endothelial cells of the brain microvasculature forming the BBB (Jefferies et al., 1984). The TfR has therefore been widely explored as a target for specific delivery of drugs to the brain by attempting to use Tf or antibodies specifically targeting this receptor (Duck and Connor, 2016; Friden et al., 1991; Gosk et al., 2004; Johnsen and Moos, 2016; Lee et al., 2000; Moos and Morgan, 2001). This suggested a TfR-mediated transcytosis pathway instead of the endocytosis pathway observed in other cells. The first observation of TfR-mediated transcytosis was published in 1987 by *Fishman and colleagues* (Fishman et al., 1987). They studied the distribution of <sup>125</sup>I-labelled Tf in the brain and its microvasculature, and demonstrated that even though most labelled Tf remained within the vasculature, intact Tf was also present in the brain parenchyma, indicating transcytosis of Tf across the brain endothelium (Fishman et al., 1987).

In studies using a radiolabeled antibody (clone OX26), which specifically targets the rat TfR without interfering with the binding site for Tf, passage across the BBB was also observed (Friden et al., 1991; Lee et al., 2000; Pardridge et al., 1991). Additionally, it was shown that when the antibodies were coupled to a therapeutic protein, increased amounts of this therapeutic protein entered the brain parenchyma in comparison to one not coupled to an antibody. These studies also observed a large retention of the complexes within the brain vasculature (Duck and Connor, 2016; Pardridge et al., 1991; Y. Zhang and Pardridge, 2001a), which was also the main observation in subsequent studies by *Moos and Morgan*, when they explored the transcytosis mechanisms of the TfR (Gosk et al., 2004; Moos and Morgan, 2001). Based on the capillary depletion technique and immunohistochemical mapping, they observed large retention of the OX26 antibody within the BCECs with only a small fraction present in the neurons situated periventricularly. It was concluded that the

amount of OX26 accumulation in the brain parenchyma was too small to justify the conclusion that the TfR undergoes transcytosis to deliver the OX26 antibody across the BBB (Moos and Morgan, 2001). They additionally based these conclusions on previous observations on much higher amounts of iron entering the brain compared to Tf (Moos and Morgan, 2000; C. M. Morris et al., 1992; Taylor et al., 1991; Ueda et al., 1993), indicating a lack of transcytosis of Tf by the TfR. This points towards the normal pathway in other types of cells where only iron is exported from the endocytosed vesicles, while the Tf is recycled with the TfR to the cell membrane (Moos and Morgan, 2001). In addition, the presence or lack of the divalent metal transporter 1 (DMT1) in the brain endothelial cells have been a matter of debate in regards to understanding the mechanisms of the TfR at the BBB (Burdo et al., 2001; Moos et al., 2006; Skjørringe et al., 2015). Inside the CNS, DMT1 expression has been confirmed in neurons, oligodendrocytes, astrocytes and in the ependymal epithelium, while studies on its expression within the brain endothelium have resulted in conflicting observations (Duck and Connor, 2016). One group was not able to confirm the expression of DMT1 in the microvessels but found expression in the neurons, despite the use of three different anti-DMT1 antibodies targeting different epitopes of DMT1 (Moos et al., 2006; Moos and Morgan, 2004). Additionally, gene expression analysis failed to confirm the presence of DMT1 in brain endothelial cells from both rat and mice brains (Enerson and Drewes, 2006; Gunshin et al., 1997; Skjørringe et al., 2015). Recently, however, the expression of DMT1 was confirmed in isolated brain capillaries, in primary isolated BCECs cultured under in vivo-like conditions, and in immortalized endothelial cells (Burkhart et al., 2016; Chiou et al., 2018a; Klomp et al., 1996; McCarthy and Kosman, 2013; 2012).

Two different pathways of TfR-mediated iron delivery to the brain have therefore been proposed (Figure 3) (Khan et al., 2018). The first involves the previously described pathway of TfR-mediated endocytosis, where holo-Tf binds to the TfR and becomes internalized into endosomes (Roberts et al., 1993). Inside the endosomes, iron detaches from Tf due to the lowering of the pH, leaving only apo-Tf bound to the TfR. The released iron is reduced to ferrous iron and translocated to the cytoplasm by DMT1. The apo-Tf together with the TfR recycle to the apical membrane or become stored inside the BCEC (Chiou et al., 2018a). Iron residing in the cytoplasm can be used by the BCECs, stored in ferritin, or exported by ferroportin residing at the basolateral membrane in collaboration with ferroxidases that oxidize iron back to its ferric state. Inside the brain parenchyma, iron is bound to Tf, or taken up by the cells of the CNS (Duck and Connor, 2016; Moos et al., 2007; Skjørringe et al., 2015). Tf is secreted locally in the CNS by oligodendrocytes and the choroid plexus epithelial cells (Aldred et al., 1987; Bloch et al., 1985; Dickson et al., 1985; Dziegielewska et al., 1984; Moos and Morgan, 2000) with indications of Tf production in BCECs as well (Chiou et al., 2018a; Connor et al., 2011; Duck et al., 2017). The expression of the iron-related proteins at the neurovascular unit was recently examined showing that the ferric reductases expressed by the BCECs in vivo and in vitro were Steap 2 and 3 (Burkhart et al., 2016). Expression of the major ferrireductase in the duodenum, Dcytb, was also reported for immortalized human brain microvascular endothelial cell line, indicating that Dcytb might provide

ferrireductases activity at the BBB (McCarthy and Kosman, 2012). Additionally, BCECs, astrocytes and pericytes all provide the activity necessary for oxidation of ferric iron to facilitate the release of iron from ferroportin. The identified ferroxidases were ceruloplasmin (both the soluble and the GPI-anchored form), and hephaestin. The expression of the ferroxidase proteins was highest in the astrocytes and pericytes indicating that these cells might be the main sources of ferroxidase activity (Burkhart et al., 2016; Klomp et al., 1996; McCarthy and Kosman, 2013; B. N. Patel and David, 1997).

The other theory involves transcytosis of the Tf-TfR complex across the BCECs without the involvement of DMT1. Again, holo-Tf binds to the TfR, which triggers its internalization into endosomes. These endosomes traverse the cell and docks at the abluminal membrane, where either iron or holo-Tf is proposed to be released into the brain parenchyma (Moos et al., 2007; Skjørringe et al., 2015). This theory, however, is also a matter of debate due to several flaws (Simpson et al., 2015). The amount of iron transported in to the brain is much higher than that of Tf (Moos and Morgan, 2000; C. M. Morris et al., 1992; Taylor et al., 1991; Ueda et al., 1993), suggesting that only iron is transported into the brain parenchyma, while Tf recycles to the plasma. Instead of Tf transport from blood to the brain, Tf is synthesized locally within the CNS by the choroid plexus cells and oligodendrocytes (Aldred et al., 1987; Bloch et al., 1985; Dickson et al., 1985; Dziegielewska et al., 1984). Therefore, Tf probably remains bound to the TfR if iron is released. Iron detaches from the Tf-TfR complex either inside the acidic endosomes due to loss of affinity to Tf at low pH, or when the Tf-TfR complex docks at the abluminal surface. Since the neutral pH of the brain interstitium does not favor the release of iron from holo-Tf, theories on how iron is released at the abluminal membrane, includes the secretion of factors like citrate or ATP from the astrocytes (Duck and Connor, 2016; Moos et al., 2007). The transcytosis theory also has difficulties in explaining, how the BCECs acquire iron for their own need (Simpson et al., 2015). The expression level of the iron-storage protein ferritin is high in the BBB, suggesting that the BBB can act as an iron reservoir, especially in the developing brain (Duck and Connor, 2016; Moos, 1995; Simpson et al., 2015). Additionally, studies using rats with mutations of DMT1 reveals a much lower level of iron within the brain and high levels of holo-Tf in plasma (Burdo et al., 1999; Simpson et al., 2015), pointing to an important role for DMT1 in the transport of iron across the BBB. In addition to the fact that BCECs are able to accumulate iron on their own, this suggests that the transport of iron across the BBB is not through transcytosis of Tf (Duck and Connor, 2016; Simpson et al., 2015; Skjørringe et al., 2015). These findings have recently been corroborated in studies performed in stem cell-based *in vitro* models of human BBB (Chiou et al., 2018a; Chiou and Connor, 2018; Chiou et al., 2018b), where the importance of DMT1 in the iron transport into the brain was underscored. Interestingly, the authors found that the main fraction of iron transported to the brain was protein-bound *and* sensitive to DMT1 inhibition, hereby suggesting a model where the iron atoms dissociate from the Tf protein, after which it can be pumped out of the BCEC by ferroportin *or* associate with the intracellular Tf pool to become secreted to the brain as protein-bound iron (Figure 3) (Chiou et al., 2018a). Free iron released to the brain parenchyma can associate with parenchymal Tf protein, possibly

synthesized by the BCECs themselves (Connor et al., 2011; Duck et al., 2017). Furthermore, an interesting function of ferritin in mediating transport of iron to the brain was also described, highlighting the complexity of the iron transport model at the BBB (Chiou, Neal, Bowman, Lippmann, Simpson, and Connor, 2018a; Montemiglio *et al.*, 2019). Such function of ferritin was recently exploited for drug delivery purposes too (Chen et al., 2017; Fan et al., 2018; Zhai et al., 2018). Thus, these recent findings support the notion that the BCECs do not simply represent conduits for iron transport to the brain, but rather participate actively by regulating both the uptake and transendothelial transport of free or protein-bound iron (Chiou et al., 2018a; Duck and Connor, 2016; Simpson et al., 2015).

### 3. The TfR as a target for brain drug delivery

In recent years, a large focus has been on the development of biological therapeutics for treating CNS-related disorders. For example, several pharmaceutical and biotechnology companies have pursued antibody variants targeted to the  $\beta$ -amyloid plaques associated with Alzheimer's disease (e.g. solanezumab (Doody et al., 2014), bapineuzumab (Salloway et al., 2014a), crenezumab (Cummings et al., 2018), gantenerumab (Ostrowitzki et al., 2017), and aducanumab (Sevigny et al., 2016)), or  $\alpha$ -synuclein associated with Parkinson's disease (e.g. prasinezumab (Jankovic et al., 2018)(NCT03100149) and BIIB054 (NCT03318523)), and the therapeutic efficacy of each antibody was shown continuously in preclinical models (Sevigny et al., 2016). However, while several of the antibodies for the treatment of Alzheimer's disease were successfully progressed into clinical trials, all of the prominent projects either failed or were halted in phase 3, largely due to futility (Biogen, 2019; Doody et al., 2014; "Lilly Announces Top-Line Results of Solanezumab Phase 3 Clinical Trial | Eli Lilly and Company," 2016; Roche, 2019; Salloway et al., 2014b). Specialized trials in different subpopulations of Alzheimer's disease have also provided negative findings (Honig et al., 2018; Liu-Seifert et al., 2015; Siemers et al., 2016), and a highly anticipated project regarding an antibody derived from drug discovery in very old people without dementia also recently failed (Biogen, 2019; Servick, 2019). Eisai's BAN2401 is currently being tested in a phase 3 trial in patients with mild Alzheimer's disease and cognitive impairment (Logovinsky et al., 2016; Satlin et al., 2016; Tucker et al., 2015). A common denominator of all of these failed clinical trials was that brain accumulation of the therapeutic antibody depended on a passive mechanism of uptake across the BBB. The resulting level of transport is therefore expected to be low, which may have decreased the therapeutic efficacy of an otherwise very potent drug. There are several ways of increasing the drug exposure in the brain, but for biological (large molecule) therapeutics transport via receptor or transporter proteins on the surface of BCECs is preferable (Preston et al., 2014). As mentioned previously, the TfR is the most widely used target for this purpose, and may have the potential of increasing the efficacy of the already existing antibody-based drugs against neurodegenerative diseases, e.g. via bi-specific antibody designs (see below).

The relevance of using the TfR as a target for drug delivery has remained largely unquestioned for several decades. The original interest in this receptor system was based on the observation that endothelial

cells of the brain expressed TfRs as opposed to endothelial cells in other organs (Jefferies et al., 1984). While this does not mean that the TfR is a receptor molecule only of the brain, these observations suggested that the selective expression in the brain capillaries could lead to preferential accumulation of TfR-targeted substances in the brain to a larger degree than for those more ubiquitously expressed in the systemic vasculature (Friden et al., 1991; Jefferies et al., 1984). In the years following this observation, the TfR gained more and more attention because seminal papers by *Friden and colleagues* showed that molecules (e.g. antibodies) directed against this receptor could efficiently accumulate along the brain capillaries, and, more importantly, could be transported across the endothelial layer of the BBB and enter the brain parenchyma (Friden et al., 1991; 1992; 1993). This sparked the hope that this receptor system could be a passageway to circumvent the low permeability of the BBB and stimulated the idea that drugs could be attached to TfR-binding molecules and be delivered into the brain in a ‘Trojan horse’-like fashion (Boado and Pardridge, 2011). In the almost 30 years that have passed since these initial findings, the research field interested in the TfR as a brain drug delivery target has grown dramatically with new studies being published on the subject with high frequency. Therefore, the amount of accumulated data is large with most of it suggesting the system to work efficiently (Johnsen and Moos, 2016; Pardridge, 2014). Many studies report therapeutic outcomes, whereas others systematically quantify the amount of substance transported into the brain (van Rooy et al., 2011a; 2011b; Y. Zhang et al., 2003b; Y. Zhang and Pardridge, 2001a). While these absolute amounts of drug transported to the brain parenchyma most often fall way below 1 % of the injected dose per gram (%ID/g), it seems to be sufficient for treatment success in preclinical models of disease (Johnsen and Moos, 2016). However, despite these large amounts of encouraging results, there has only been very little translation of these findings into a clinical setting (Okuyama et al., 2019), and thus, there is a clear need for more data shedding light not only on the therapeutic outcomes in preclinical models of disease but also on the mechanistic aspects of the transport process, which may provide valuable insight as to why many current strategies have failed so far. In addition, potential publication bias may also result in negative findings never being published, hereby making important information unavailable for the broader research community. Thus, several aspects pertaining to the use of the TfR as a target for brain drug delivery need to be clarified before clinical use can become a realistic ambition.

In the next sections, we review the use of TfR as a target for drug delivery in the context of antibody-based medicines and nanocarriers. The underlying literature foundation was derived from several PubMed search strings using the search criteria “transferrin receptor AND brain AND drug delivery” or “transferrin receptor AND (brain OR “central nervous system”)” (last search was performed on July 10<sup>th</sup>, 2019). The PubMed search yielded a total of 286 and 1178 papers, respectively, of which 216 papers were sorted with respect to type of drug delivery system (i.e. antibodies or nanocarriers) and used for the individual sections below. The following sections will first describe the different ligands and monoclonal antibodies used to target the TfR at the BBB and investigate how the functionality of these different ligands depict treatment outcome when they carry therapeutically active molecules as their cargo. Additionally, the different kinds of

nanocarriers that have been functionalized with TfR-targeting ligands are described (e.g. liposomes and polymer nanoparticles). Lastly, we come with suggestions on how to modify the different drug delivery strategies to improve the subsequent transport into the brain. These include modifications of affinity and avidity in antibody-based medicines, as well as the addition of cell-penetrating peptides or stimuli-sensitive drug release in nanocarrier systems.

### 3.1. Ligands

The choice of ligand in TfR-targeted drug delivery system varies greatly across studies. It encompasses both the endogenous ligand, Tf, together with several different antibodies and peptides that bind to different epitopes of the receptor molecule. The endogenous Tf molecule has been used extensively throughout many studies in the past 30 years. It has the advantage that it can bind to the ‘normal’ binding pocket on the TfR molecule and initiate the normophysiological mechanism of endocytosis of the Tf-TfR complex into the BCEC. This endocytosis process will likely be affected by the size of the drug or nanocarriers attached to the Tf molecule, where endothelial uptake of larger structures may distort the normal intracellular sorting mechanism (Freskgård and Urich, 2017; Fullstone et al., 2016). As described earlier, the main body of data on the dynamics of the TfR in the BCECs suggests that neither the receptor nor the Tf molecule transcytoses into the brain parenchyma from the luminal side of the vessel (Duck and Connor, 2016; Simpson et al., 2015; Skjørringe et al., 2015). Instead, the iron is released from its binding to Tf, after which it is pumped out of the endosome and subsequently out of the BCEC (Skjørringe et al., 2015). Since the Tf molecule has allowed for drug accumulation in the brain parenchyma using a wide variety of constructs and nanocarriers, there must therefore be a mechanism, which can release the drug or nanocarrier from the TfR and transport it inwards to the brain parenchyma (Boado and Pardridge, 2011; Johnsen and Moos, 2016; Wiley et al., 2013). This mechanism has yet to be described (Johnsen et al., 2017).

Another widely used TfR-targeting molecule is the OX26 antibody, which recognizes the rat TfR. OX26 was originally described by *Jefferies and colleagues* to specifically bind the rat TfR with high affinity without interfering with the normal binding site of endogenous Tf (Hill et al., 1985; Jefferies et al., 1984; 1985). It was also found to label the TfR in various tissues, but not along the blood vessels in them, and it was used to underscore the observation that TfRs could only be detected in capillaries of the brain (Friden et al., 1991; Jefferies et al., 1984). Within a few years, OX26 was tested for its ability to be transported across the BBB, and the positive findings of these investigations sparked a long-lasting claim that due to the successful transport of the OX26 antibody, the TfR must also have been transcytosed (Bickel et al., 1993; Friden et al., 1993; 1991). Like for the endogenous Tf molecule, OX26 has now been tested as a targeting molecule, both as fusion constructs with different drugs and together with different kinds of nanocarriers, leading to therapeutic efficacy of several preclinical models of brain disease, including Parkinson’s disease (Pardridge, 2005; Y. Zhang et al., 2003b; 2004a), Alzheimer’s disease (Y. Saito et al., 1995), stroke (Song et al., 2002; Y.

Zhang and Pardridge, 2006; 2001a; 2001b), and especially the brain cancer type, glioblastoma multiforme (GBM) (Xia et al., 2007; Y. Zhang et al., 2003a; 2004b; 2002), which will be elaborated in the next sections. Murine analogous variants of OX26 (i.e. antibodies with high affinity against the murine TfR) have also been produced (e.g. clones 8D3 and RI7217), and the existing data suggests both transcytosis or inability to do so (Bourassa et al., 2019; Jeong Lee and Pardridge, 2000; Lee et al., 2000; 2002; Manich et al., 2013; Paris-Robidas et al., 2016; 2011; Y. Zhang and Pardridge, 2005). It should be noted that determining transcytosis is still a big methodological challenge, since techniques like brain capillary depletion could overestimate the true level of transport (Freskgård et al., 2014; Watts and Dennis, 2013). However, there has been good progress in validating transport of TfR-targeted brain medicines, especially when binding to intraparenchymal targets can be specifically determined (Sehlin et al., 2016).

In addition to using endogenous Tf or antibodies, some groups have developed small peptides that bind to the TfR (Kuang et al., 2016; Z. Wang et al., 2015). The advantages of such small peptides are that the resulting construct does not compete with the endogenous Tf for binding to the receptor, and that potential side effects imposed by the presence of the Fc effector domain of the antibody are avoided (Couch et al., 2013). However, peptides are known to be very unstable in plasma and bind their target with very low affinity, which may make them less relevant compared to antibodies. Also, in comparison with the two abovementioned strategies of TfR-targeting, there is less data on the use of peptides for this purpose, although the popularity is increasing (Bi et al., 2016; L. Cui et al., 2018; Kuang et al., 2013; Liang et al., 2018; Prades et al., 2012; Z. Wang et al., 2015; Zong et al., 2014). For example, a recent study compared the functionality of different peptides targeting the TfR and low-density lipoprotein receptor-related protein 1 (LRP1) on polymer nanoconjugates showing that the choice of peptide to target the TfR is important for the subsequent uptake potential in the brain (Israel et al., 2019). Even less information is available regarding the use of aptamers as ligands for TfR-targeted brain drug delivery (Macdonald et al., 2017; 2016; McConnell et al., 2018; C. Mu et al., 2013).

### 3.2. Drug conjugates targeting the TfR

The use of the TfR as a target for brain drug delivery using conjugates of a drug and a targeting molecule was initiated by *Friden and colleagues* in the early 1990s (Friden et al., 1991). Using methotrexate as a model drug, they could show that attachment to the OX26 antibody increased the brain parenchymal levels of this molecule substantially compared to free drug, although the absolute quantity of methotrexate in the brain after employment of this targeting strategy was still below 0.3 %ID (Friden et al., 1991). These studies indicated that small molecule drugs could be transported into the brain using a TfR-targeting strategy but did not address the possibility of transporting larger molecules like proteins across the BBB. This was proven shortly after in a study showing efficient brain delivery of nerve growth factor (NGF) conjugated to an anti-TfR antibody (Friden et al., 1993). The transport of NGF across the BBB was shown to increase the survival of both

cholinergic and non-cholinergic neurons, which is of high relevance in treatments for Alzheimer's disease (Bäckman et al., 1995; 1996; Friden et al., 1993; Granholm et al., 1994). The construct also improved the therapeutic outcome in a model of Huntington's disease and after NMDA-induced brain lesions (Charles et al., 1996; Kordower et al., 1994), and spinal cord motor neuron function after delivery of glial-derived neurotrophic factor (GDNF) instead of NGF (Albeck et al., 1997). Attempts were also made to develop anti-TfR antibodies for use in primates and humans (Friden et al., 1996; Walus et al., 1996), and constructs were designed with endogenous Tf as a targeting ligand (Hagihara et al., 2000; Laske et al., 1997; X. B. Li et al., 2000; Martell et al., 1993; Weaver and Laske, 2003). While most of the transport of OX26 across the BBB was ascribed to receptor-mediated transcytosis with the TfR like what was observed for Tf, some claimed that the co-localization of OX26 with the Golgi apparatus suggested the transport pathway to be adsorptive-mediated transcytosis (Broadwell et al., 1996). In parallel with these findings and in the following years, *Pardridge and colleagues* published a series of studies where they investigated the possibility of using the TfR as a target for BBB transport, especially using the OX26 antibody (Bickel et al., 1993; Y. S. Kang et al., 1994; Y. S. Kang and Pardridge, 1994; Lee et al., 2002; 2000; Pardridge et al., 1995; 1994; 1998; Shin et al., 1995; Song et al., 2002; Wu et al., 1996; Wu and Pardridge, 1999; 1998; 1996; Wu et al., 2002; Y. Zhang and Pardridge, 2001a). Like *Friden*, *Pardridge* showed improved brain uptake of OX26, and provided evidence on how to conjugate this antibody to different molecules including brain-derived neurotrophic factor (BDNF) (Pardridge et al., 1998; 1994; Y. Zhang and Pardridge, 2006; 2001b), epidermal growth factor (EGF) (Deguchi et al., 1999), fibroblast growth factor (FGF) (Song et al., 2002), vasoactive intestinal peptide (VIP) (Bickel et al., 1993), antisense oligonucleotides (Y. S. Kang et al., 1995; Wu et al., 1996; Xia et al., 2007), peptide nucleic acids (PNAs) (Pardridge et al., 1995), and different therapeutic peptides (Bickel et al., 1995; Y. Saito et al., 1995). Furthermore, the pharmacokinetic profile of the different constructs was described (Wu et al., 2002; Wu and Pardridge, 1998), as well as the importance of choosing either carboxy or amino terminal coupling to the therapeutic protein (Pardridge et al., 1998). Most prominently, these constructs could provide neuroprotection in cerebral ischemia and stroke models in the rat (Song et al., 2002; Wu and Pardridge, 1999; Y. Zhang and Pardridge, 2006; 2001b; 2001a), and the findings of efficient transport into the brain could largely be recapitulated in the mouse using either the 8D3 or RI7217 antibodies (Boado et al., 2009; Jeong Lee and Pardridge, 2000; Lee et al., 2000; Y. Zhang and Pardridge, 2005). Transendothelial transport was also shown by others for 8D3 reconfigured to be a  $\beta$ -amyloid PET tracer (Hultqvist et al., 2017; Sehlin et al., 2016; Syvänen et al., 2017). Later, the *Pardridge* group developed single chain variable fragments (scFv) and chimeric anti-TfR antibodies, which could be fused to numerous different therapeutic proteins (Boado et al., 2009; 2000; J. Y. Li et al., 1999). For example, a genetically engineered chimeric anti-TfR antibody could deliver erythropoietin (EPO) (Chang et al., 2018), N-sulfoglucosamine sulfohydrolase (SGSH) (Boado et al., 2018), tumor necrosis factor receptor (TNFR) (Chang et al., 2017; Sumbria et al., 2013a; 2013b; Zhou et al., 2011a; 2011d), GDNF (Fu et al., 2010; Sumbria et al., 2013a; Zhou et al., 2011b; 2010), iduronate-2-sulfatase

(IDS) (Zhou et al., 2012),  $\alpha$ -L-iduronidase (IDUA) (Boado et al., 2011), anti-survivin siRNA (F. Wang et al., 2011), and anti- $\beta$ -amyloid scFvs (Boado et al., 2010; Zhou et al., 2011c). Of note, the brain uptake of these different constructs based on a chimeric antibody (Boado et al., 2010; Zhou et al., 2012; 2011c; 2011a; 2010) differed substantially compared to constructs based on OX26 (Y. S. Kang et al., 2000; Pardridge et al., 1998; Y. Saito et al., 1995) (2.2 – 3.5 %ID/g versus 0.12 – 0.15 %ID/g, respectively), showing that despite all antibodies having high affinity towards the TfR, the resulting transport cannot be expected to be similar ( $K_D$  ranging between 96 pM – 5 nM (Boado et al., 2011; 2010; 2009; Thom et al., 2018; Zhou et al., 2012; 2011d; 2011a; 2010)). In addition to the choice of therapeutic entity, redox-sensitive linkers were also incorporated to enable activation or release of the therapeutic entity from the targeting ligand (Bickel et al., 1995; Y. S. Kang et al., 2000). Furthermore, results from a recent phase 1/2 trial showed that high affinity targeting of anti-TfR antibodies fused to IDS could reduce CSF levels of heparan sulfate in patients suffering from mucopolysaccharidosis II (MPSII), illustrating that TfR-targeted brain drug delivery is a reasonable strategy for treatment of brain-related disorders (Okuyama et al., 2019; Sonoda et al., 2018; Tanaka et al., 2018).

Despite the vast amounts of evidence presented for the strategy of targeting the TfR with high affinity antibodies, the essence of these findings have been questioned by numerous groups (Manich et al., 2013; Moos and Morgan, 2001; Niewoehner et al., 2014; Paris-Robidas et al., 2011; Yu et al., 2011). The effective transport of OX26 antibodies across the BBB was first questioned by *Moos and Morgan* (2001), who showed that it remained confined in the BCECs of the capillary wall with little evidence of transport into the brain parenchyma (Moos and Morgan, 2001). This highlighted the possibility that the OX26 antibody (and other analogous high affinity antibodies against different species) would be so tightly bound to the TfR that release during the endosomal sorting process was unlikely (Dennis and Watts, 2012; Freskgård and Urich, 2017). Similar observations were also obtained in the mouse, again suggesting that the antibody could not be detected in post-vascular compartments of the brain after intravenous administration (Alata et al., 2014; Cabezón et al., 2017; 2015; Manich et al., 2013; Paris-Robidas et al., 2016; 2011; Webster et al., 2017). While these findings were discouraging for the field of TfR-targeted brain drug delivery, they did point towards the option that the distorted transport of antibodies into the brain could be recovered by modulating the binding mode between the antibody and the TfR (Lichota et al., 2010). A decade later, the work by *Yu et al.* showed that the binding mode could indeed be modified by decreasing the affinity of the antibody towards the TfR (Yu et al., 2011). This led to a higher accumulation of antibodies in the brain parenchyma (0.2 – 0.6 %ID/g), and could also significantly reduce the expression of the Alzheimer's disease-related enzyme, beta-secretase 1 (BACE1), with a bi-specific antibody targeting both the TfR and the BACE1 enzyme (Yu et al., 2011). These results were corroborated in subsequent studies showing that the reason for the low affinity antibodies accumulating in the brain was because they could circumvent intracellular sorting targeted for degradation in the lysosomes, a location shown to be the main accumulation site for the high affinity antibodies (Bien-Ly et al., 2014) and liposomes functionalized with high affinity antibodies (Johnsen et al., 2017). Although difficult to directly

prove experimentally, this data suggested that the TfR-targeting molecule needs to be able to dissociate from the receptor during the endosomal sorting process, because too long occupancy on the receptor is a signal for the cell to target the strongly binding molecules towards degradation in the lysosomes (Freskgård and Urich, 2017; Watts and Dennis, 2013). Related to this, strong indirect evidence has been provided to suggest that antibody constructs with proper design can be released from the TfR, enter the brain, and bind to an intraparenchymal target, hereby underscoring that the antibody binding mode interferes with the transport process (Hultqvist et al., 2017; Niewoehner et al., 2014; Sehlin et al., 2016; Yu et al., 2011). In addition to affinity, others have suggested that the binding mode also could be changed either by modulating the avidity (Hultqvist et al., 2017; Niewoehner et al., 2014; Yu et al., 2014), engineering dual variable domain antibodies (Karaoglu Hanzatian et al., 2018), or introduce pH-sensitivity in the variable domain of the TfR-targeting antibody (all explained in detail later)(Sade et al., 2014). In addition to using antibodies for transporting drugs across the BBB, different conjugates have been made of endogenous Tf and molecules such as horseradish peroxidase (HRP, as a model drug) or nucleic acids for therapeutic gene expression in the brain parenchyma (Osborn et al., 2008; Roberts et al., 1993; Visser et al., 2004a). The observed efficiency of Tf in transporting drugs has also been utilized by conjugating the protein with diphtheria toxin for cytotoxic effects in brain cancer (Weaver and Laske, 2003). This construct was taken into clinical trials, although it was halted at phase 3 (NCT00083447). Interestingly, only a few studies have investigated constructs of drug-conjugated TfR-targeted peptides; however, it was shown that the T7 peptide could increase the brain accumulation of shRNA to mediate vascular endothelial growth factor (VEGF) knockdown for GBM treatment (Kuang et al., 2016).

### 3.3. Liposomes

With increasing amounts of data suggesting the TfR to be a relevant target for brain drug delivery published through the first part of the 1990s, some researchers started to investigate the possibility of attaching larger nanocarriers to the different TfR-targeting molecules to increase the brain concentration of the cargo encapsulated in the nanocarriers. Like for the TfR-targeted drug constructs mentioned above, this field was also pioneered by the studies of *Pardridge and colleagues*, who in the mid 1990s showed that this concept could indeed be feasible (Huwyler et al., 2002; 1996; Shi et al., 2001a; Shi and Pardridge, 2000; Shi et al., 2001b; Y. Zhang et al., 2004b; 2004a; Y.-F. Zhang et al., 2003). Using the OX26 antibody as the targeting molecule, liposomes containing daunomycin were administrated to rats, and the daunomycin cargo could after a period of circulation clearly be found associated with the brain (Huwyler et al., 1996). The first studies did, however, not provide any evidence of effective BBB passage, but instead showed that the brain association of daunomycin increased with tuning of the antibody density on the liposomes surface (Huwyler et al., 1996). Interestingly though, the fraction of drug that reached the brain was an order of magnitude lower than what was previously reported for the OX26 drug constructs alone (Johnsen and Moos, 2016). This suggested that the transport of larger nanocarriers is much less efficient than antibodies alone, although it may be

compensated by the fact that nanocarriers can carry a large drug load (Huwlyer et al., 1996). Conversely, liposome delivery with an efficiency comparable to OX26 alone has also been reported (Y.-S. Kang et al., 2016). These preliminary findings were later expanded upon in subsequent studies using the same OX26-functionalized liposomes administered to monolayers of the immortal rat BCEC line, RBE4 (Cerletti et al., 2000; Huwlyer et al., 2002). In these experiments, the liposomes were found to transcytose, as was also expected to happen in vivo, and they could also protect P-gp substrates from efflux during endocytosis into the BCECs (Cerletti et al., 2000; Huwlyer et al., 2002). Later, different liposomal formulations were tested for encapsulation of nucleic acids, i.e. by including positively charged phospholipids, which allowed for having both plasmid DNA or small RNAs as the drug cargo (Shi et al., 2001b; 2001a; Shi and Pardridge, 2000; Y. Zhang et al., 2004b; 2004a; Y.-F. Zhang et al., 2003). With this drug delivery strategy, it was possible to induce expression of  $\beta$ -galactosidase in the brain after administering the liposomes intravenously, and the treatment could be specified to the brain by having the plasmid DNA product expressed under the control of a glial fibrillary acidic protein (GFAP) promoter instead of the more classical SV promoter (Y. Zhang et al., 2004a; 2003b; Zhao et al., 2010; Zhu et al., 2004). The concept also showed benefit in preclinical brain disease models, e.g. with an increase of tyrosine hydroxylase expression (due to delivery of plasmid DNA) in dopaminergic neurons in a model of Parkinson's disease (Y. Zhang et al., 2004a; Y.-F. Zhang et al., 2003), delivery of GUSB enzyme in a model of MPSII (Y. Zhang et al., 2008), and with tumor size reduction and increased survival after delivery of interfering RNAs in a model of GBM (Y. Zhang et al., 2004b). One study even showed that TfR-targeted liposomes encapsulating plasmid DNA could be administered to a pregnant mouse, cross the placental barrier and mediate gene expression in the developing brain of the fetus (Cornford et al., 2015). Furthermore, endogenous Tf was tested in several studies as a targeting molecule for liposome transport with a variety of therapeutic effects including improvement after stroke and brain injury (Omori et al., 2003; Suresh Reddy et al., 2006), increased brain uptake of HIV drugs (Prabhakar et al., 2011), tumor size reduction in GBM (Jhaveri et al., 2018; Lakkadwala and J. Singh, 2019; C. Liu et al., 2017; Salzano et al., 2016; Sonali et al., 2016b; Zheng et al., 2015), or general gene knockdown after siRNA delivery into the brain (Cardoso et al., 2010; 2008). One study used peptide-based targeting of the TfR for delivery of vinblastine in a model of GBM (L.-M. Mu et al., 2017). The efficiency of drug transport using Tf-modified liposomes has also been improved by co-attachment of different cell-penetrating peptides, e.g. TAT from HIV, which has led to major increases in the %ID/g that reaches the brain. In fact, some formulations could deliver in the order of 4 %ID/g, which is comparable or superior to what can be obtained in a non-CNS tumor model (Sharma *et al.*, 2012; 2013; 2014; 2016; Wilhelm *et al.*, 2016). Still, for mono-targeted liposomes, TfR antibodies were superior to both endogenous Tf and other relevant targets on the endothelial surface of the BBB (van Rooy et al., 2011b), although others claim that only endogenous Tf-functionalized nanoparticles will transcytose as opposed to nanoparticles functionalized with monoclonal antibodies (Clark and Davis, 2015). The size of the liposomes has also been described as a major factor for determining subsequent BBB passage, where increasing sizes

will lead to a decrease in cargo/liposome transport (Hatakeyama et al., 2004). It is therefore up for debate, whether results suggesting an accumulation of 40 %ID using liposomes between 750 – 2000 nm can be regarded as valid (Soni et al., 2005). TfR-targeted liposomes can also be found accumulating in the lysosomes of BCECs, hereby suggesting that endocytosis of such large constructs would likely distort the normal endosomal sorting process, and instead target the endocytosed material for degradation in the lysosomes (Johnsen and Moos, 2016; Visser et al., 2005). Like for the OX26 antibodies alone, the possibility of liposome transcytosis into the brain after targeting with OX26 was questioned by *Moos and colleagues*, who could only show accumulation of liposomes in the BCECs, but not in the brain parenchyma after brain capillary depletion (Gosk et al., 2004; Moos and Morgan, 2001). Later, it was found that while the liposome was confined within the vascular fraction of the brain, an encapsulated, small molecule drug could be detected in the brain parenchyma (Johnsen et al., 2017). This raises the possibility that liposomes may only be able to transport the drug cargo to the BCECs, who are then responsible for transporting the cargo further into the brain parenchyma (Johnsen et al., 2017; Johnsen and Moos, 2016). Such a release mechanism still remains to be described. Factors such as ligand density and dosing regimen of the TfR-targeted liposomes were also shown to impact the resulting brain transport (see later) (Huwlyer et al., 1996; Johnsen et al., 2017; 2019; Papadia et al., 2017). Furthermore, the impact of different variants of anti-TfR antibodies on the resulting transport of liposomes has not been assessed but could be studied in detail by exploiting new developments in nanobiotechnology (Kedmi et al., 2018).

#### 3.4. Polymeric nanoparticles

Another widely used way of composing a nanoparticle is based on polymers of macromolecules (Kreuter, 2014). These polymers can be designed to infer different capabilities to the nanoparticle, e.g. controlled release mechanisms or encapsulation of specific drug compounds. Furthermore, they are often biodegradable within a short time frame, which has been suggested as a requirement for a brain drug delivery system based on polymers (Kreuter, 2014). The field of polymeric nanoparticles using the TfR as a target for brain drug delivery is not as well-established as the field of liposomes described above. Nevertheless, polymers are important in both strategies, since almost all studies on TfR-targeted liposomes employ a polymer coating to increase the circulatory properties of the formulations, e.g. the well-known polyethylene glycol (PEG). PEG itself has been combined with chitosan to produce nanoparticles that could deliver anti-caspase peptides to the brain using the OX26 antibody as a targeting molecule (Aktaş et al., 2005; Yemisci et al., 2015; 2012). OX26 has also been used to target both poly-lactic acid (PLA)-PEG, PEG-polycaprolactone (PCL), and poly(lactic-co-glycolic acid) (PLGA) nanoparticles to the brain, hereby delivering amphotericin B, A $\beta$  peptide, temozolomide, and Tempol across the endothelial layer (Bommana et al., 2012; Carroll et al., 2010; Loureiro et al., 2016; Pang et al., 2008; Ramalho et al., 2018; Sriramoju et al., 2015; Tang et al., 2015). The anti-mouse TfR antibody variants, 8D3 and RI7, were also used to target polymer nanoparticles towards the brain (Bhattacharya et al.,

2008; Papademetriou et al., 2013). PLGA and PEG nanoparticles functionalized with endogenous Tf could deliver nevirapine, paclitaxel, temozolomide, and methotrexate to mediate therapeutic benefit in disease models both in vivo and in vitro (Atul Jain et al., 2015; Aviral Jain et al., 2011; T. Kang et al., 2015; Kuo et al., 2011; P. Zhang et al., 2012a). Peptide- and aptamer-based targeting of PEG-PLA and PLGA nanoparticles was also used to enhance brain uptake (Bi et al., 2016; Falanga et al., 2018; Gomes et al., 2017; C. Mu et al., 2013). Chitosan nanoparticles have been functionalized with several types of TfR-targeting ligands to deliver drugs across the BBB (Gu et al., 2017; Karatas et al., 2009; Sahin et al., 2017; Yemisci et al., 2018). Less used polymers include poly( $\beta$ -L-malic acid) (PbMLA) for temozolomide and anti-tumor antibody fragment delivery (Fujita et al., 2007), polyphosphoester (PPE) micelles for paclitaxel delivery and anti-GBM activity in vivo, polyamidoamine (PANAM)-PEG for RNAi delivery, luciferase inhibition, and temozolomide delivery in models of GBM model in vivo, 3-diaminobutyric polypropylenimine dendrimer (DAB) for DNA condensation and delivery in vivo, and d-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) micelles for docetaxel delivery in vivo (Agrawal et al., 2017a; 2017b; Kuang et al., 2013; Patil et al., 2010; Somani et al., 2014; Sonali et al., 2016a; T. Sun et al., 2017; P. Zhang et al., 2012b). A TfR-targeting peptide functionalized to the surface of polyanionic polymalic acid nanoconjugates in a dual-targeting setup with tre-leucine lead to an improve transport of rhodamine (model cargo) to the brain parenchyma (Israel et al., 2019). The brain uptake efficiency was, however, less consistent than what was observed for a similar nanoconjugate targeted to LRP1. Furthermore, camptothecin complexed in mucic acid polymer nanoparticles could be delivered to brain metastases of breast cancer via TfR-mediated transport facilitated by spacing the nanoparticle and Tf protein with an acid-cleavable linker (Wyatt and Davis, 2019). It thus seems clear that various kinds of polymers can mediate brain drug delivery in combination with TfR-targeting. However, this large number of different polymers currently employed as nanocarriers for TfR-mediated brain drug delivery highlights that more research is needed to validate the relevance of each type of polymer nanoparticle for use in such a drug delivery strategy. First, there is a great lack of studies reproducing earlier findings (maybe in a new context), and there is currently no head-to-head comparison of different types of polymer nanoparticles to analyze whether any specific type of polymer could be suited for brain drug delivery. Here, attention could be given to studies investigating other receptor systems for transport, as these fields may be more developed. It is also of special interest to compare different kinds of polymeric nanoparticles with liposomes to decipher whether one strategy is superior to the other. However, this will likely be context-based with variations appearing as a function of both the nanoparticle composition (i.e. what types of lipids and polymers and the molar ratio between them) and the disease in which the different strategies are compared. For a thorough review on polymers used for brain drug delivery, please refer to *Kreuter (2014)*(Kreuter, 2014).

### 3.5. Gold nanoparticles

Another type of nanoparticle that offers both the possibility of delivering drugs, but also strong advantages in basic studies of nanoparticle behavior in the brain, is the gold nanoparticle. Gold nanoparticles are produced by reduction of Au salt in solution, and this procedure gives much better control over the size and polydispersity of the resulting nanoparticles compared to both liposomes and polymeric nanoparticles (Sperling et al., 2008). Gold nanoparticles can be produced in sizes down to 1.5 nm, which is well below what can be accomplished with other types of nanoparticles (Male et al., 2016). TfR-targeting of gold nanoparticles has received increased attention through the recent years, and several studies have provided interesting evidence on the passage of different sized particles into the brain. Initial studies on the transport capabilities of the OX26 antibody and Tf were performed with the use of small diameter gold nanoparticles for tracking (Bickel et al., 1994; van Gelder et al., 1997). Prades and colleagues functionalized gold nanoparticles (5 – 13 nm) with a fusion peptide with the capacity of attaching to both the TfR and  $\beta$ -amyloid (Prades et al., 2012). These gold nanoparticles were first administrated to a primary bovine in vitro model of the BBB, where they were shown to transcytose efficiently. After intraperitoneal injection into mice, the gold nanoparticles could also be detected in neuronal cells in the brain cortex (Prades et al., 2012). Such uptake likely has a two-faced explanation with contributions both from the TfR-targeting and from the small size of the gold nanoparticles. In fact, others have shown that gold nanoparticles of such small size can enter the brain without any targeting, although introduction of a targeting ligand increases the efficiency of the transport markedly (Gromnicova et al., 2013; 2016b; Male et al., 2016). Working with larger nanoparticles, the group of Mark E. Davis has presented evidence that PEGylated gold nanoparticles of around 80 nm can be transported across the BBB and be detected by light and electron microscopy in several regions of the brain parenchyma (Wiley et al., 2013). Interestingly, the transport efficiency could be increased by tuning the avidity of the targeted gold nanoparticle, i.e. by optimizing the number of endogenous Tf molecules present on the gold nanoparticle surface (Wiley et al., 2013). This concept was applicable regardless of the initial size of the gold nanoparticle (Wiley et al., 2013). The group also showed that the gold nanoparticle could be designed to take advantage of the known uptake process of the TfR, where endocytosis and subsequent sorting will lead to acidification of the endosome. By spacing the endogenous Tf and the PEG-coating layer with a pH-sensitive linker, the authors could increase the transport of gold nanoparticles into the brain, likely because the presence of a pH-sensitive linker allowed for dissociation of the gold nanoparticle from the endocytosed receptor (Clark and Davis, 2015). However, this was only true, when the gold nanoparticles were functionalized with endogenous Tf compared to a TfR antibody. This again points towards the fact that binding to a ‘wrong’ part of the TfR may distort the intracellular sorting after endocytosis, thus, targeting the material directly for degradation in the lysosomes (Clark and Davis, 2015). Along with this, it was recently shown that gold nanoparticles functionalized with different variants of anti-TfR antibodies were transported differently across the BBB (Johnsen et al., 2018). In particular, an antibody binding the TfR with only one of its two variable domains made significant improvements to the transport efficacy, underscoring the findings of previous studies that monovalent binding

of either antibodies or endogenous Tf protein to the TfR is favorable for brain transport (Clark and Davis, 2015; Johnsen et al., 2019; 2018; Wiley et al., 2013). Smaller gold nanoparticles have also been attached to Tf peptides and dual-targeted by co-attaching EGF peptide to obtain both transfer capabilities across the BBB and accumulation properties in GBM tumors (Dixit et al., 2015a; 2015b).

Gold nanoparticles may not necessarily be the best choice for brain drug delivery since the drug loading capacity is well below that of a liposome or a polymeric nanoparticle. However, the gold nanoparticles are due to their composition, chemical properties, and easily controlled production, a good model nanoparticle to employ in basic studies of nanoparticle behavior in tissues (Kempen et al., 2013), e.g. in the brain or in the process of transcytosing into it. This was nicely illustrated in an electron microscopy-based mapping of the endocytosis process of gold nanoparticles functionalized with the 8D3 antibody (targeting the mouse TfR) (Cabezón et al., 2015). These investigations revealed that the gold nanoparticles were taken up by the BCEC in vivo, but also that the level of transcytosis observed was very low (Cabezón et al., 2015). The picture was further complicated by the fact that the few gold nanoparticles that had transcytosed seemed to be stuck in the meshwork of the basement membrane to which the BCECs anchor, an observation that was also reported for other metal nanoparticles after mannitol-induced disruption of the BCEC layer (Cabezón et al., 2015; Muldoon et al., 1999). This is in direct opposition to the studies mentioned earlier, which showed the presence of gold nanoparticles associated with neuronal cells distances away from the capillary (Clark and Davis, 2015; Johnsen et al., 2018; Wiley et al., 2013). Even though gold nanoparticles are easily visualized with electron microscopy, sample preparation for this method can induce artefacts (e.g. salt crystals) that are indistinguishable from the real gold nanoparticles (Johnsen, 2018). It is therefore important to validate the findings by doing elemental composition analysis on the area of interest using energy-dispersive X-ray spectroscopy (Johnsen, 2018), especially when indicating transcytosis based on observations of gold nanoparticles in parenchymal structures like neural processes (Johnsen et al., 2018). Lastly, the presence of gold nanoparticles in a tissue sample is also easily detectable using methods like inductively-coupled plasma-mass spectrometry (ICP-MS), whereby quantitative data on the whole nanoparticle in a tissue fraction (and not just a cargo) can be obtained (Johnsen et al., 2019; 2018; Male et al., 2016). These factors represent a major advantage in the studies on the transport of nanoparticles into the brain.

### 3.6. *Miscellaneous nanoparticles*

The nanoparticles described above account for the clear majority of studies currently published using the TfR as a target for brain drug delivery. However, other (and less well known) kinds of nanoparticles have also been tested in this context. For example, carbon dots attached to endogenous Tf could be transported into human brain tumor cells and the brains of zebrafish, the lowest species known to have a functional BBB (L. Li et al., 2016; S. Li et al., 2016). Quantum rods attached to endogenous Tf could deliver saquinavir in a model of neuroAIDS, whereas attachment of quantum dots to RI7217 antibodies underscored the fact that no

transcytosis is taking place for these kinds of strongly binding antibodies (Mahajan et al., 2010; Paris-Robidas et al., 2016). RI7217 binds to the mouse TfR with a  $K_D = 6$  nM (Johnsen et al., 2018). PEGylated graphene oxide nanoparticles functionalized with Tf delivered doxorubicin and improved the treatment outcome in a model of GBM (G. Liu et al., 2013). Ferritin nanocages were found to transport across the BBB to deliver therapeutic cargo in several models of GBM, and their transport was hypothesized to be dependent on binding to TfRs (Chen et al., 2017; Fan et al., 2018; Zhai et al., 2018). This hypothesis was recently validated in a study showing transcytosis of ferritin as a means of transporting iron atoms from the blood to the brain (Chiou et al., 2018a; Chiou and Connor, 2018), and the structure of the ferritin-TfR complex was recently published (Montemiglio et al., 2019). Magnetic nanoparticles either free or encapsulated in liposomes also benefit from being functionalized with endogenous Tf (Ding et al., 2014; Yan et al., 2013). One study showed that even though external magnetic force was applied to a specific area, the system still needed the TfR targeting for the magnetic nanoparticles to move across the BBB (Ding et al., 2014; Yan et al., 2013). Another study functionalized polymer nanoparticles with small magnetic nanoparticles and OX26 antibodies, and found this to be beneficial for cellular uptake in SHSY-5Y cells (N. Cui et al., 2018). Albumin nanoparticles targeted towards the TfR either with OX26 or RI7217 antibodies could mediate improved anti-nociceptive effects of loperamide in mice (Ulbrich et al., 2009). That such effects can be mediated in mice using OX26 as a targeting molecule with equal efficiency to RI7217 is, however, perplexing (Boado et al., 2009). OX26 was raised in the mouse against the rat TfR, it binds only weakly to the mouse variant with a  $K_D = 16$   $\mu$ M, and OX26 immunoliposomes associate only very slightly to bEnd.3 cells in vitro (Johnsen et al., 2018; 2017). Suicide gene delivery via phage nanoparticles was used to reduce the volume of intracranial xenograft tumors after targeting with iron mimic peptides that could exploit the normal sorting pathway of the TfR (Staquicini et al., 2011). In addition, mesoporous silica nanoparticles endowed with Tf on their surfaces could traverse the BBB both in vitro and in vivo (in zebrafish and mice) (Heggannavar et al., 2018; Yang et al., 2016). Finally, different types of lipoprotein particles (including LDL and HDL) has been functionalized with TfR-targeting peptides to increase the uptake of therapeutic cargo (including vincristine and camptothecin) in C6 rat xenograft GBM tumors established in mice (L. Cui et al., 2018; Liang et al., 2018).

#### **4. Challenges for current TfR drug delivery strategies**

##### *4.1. Does the TfR transcytose drugs into the brain?*

Regardless of the disagreement on whether a TfR-targeted drug delivery system will transcytose through the BBB, the vast amount of data showing a preclinical therapeutic efficacy benefit suggest that in some form the TfR is a good choice for transporting drugs into the brain parenchyma. However, claiming that any therapeutic effects are obtained by transcytosing the TfR with a drug is still controversial. As described previously, the overwhelming evidence on the physiological dynamics of the TfR in brain iron uptake suggests endocytosis

of the receptor, followed by a dissociation of iron from the Tf-TfR complex (Burkhart et al., 2016; Duck and Connor, 2016; Hersom et al., 2016; Skjørringe et al., 2015). In relation to this, one could ask the question: Why would the endothelial cells of the brain have developed an iron uptake mechanism (i.e. transcytosis) so different compared to other cells, especially those also composing a biological membrane such as the epithelial lining of the gut? What would have been the evolutionary driver?

Recently, the TfR was detected on the abluminal membrane of the BCECs *in vivo*, but these findings were interpreted in the context that BCECs can act as a reservoir and regulator of iron uptake and clearance in the brain (Simpson et al., 2015). This means that by expressing TfRs on the abluminal membrane, the BCEC could 'sense' the intraparenchymal iron status and regulate the recycling rate of TfRs on the luminal membrane to either increase or decrease iron-Tf uptake from the blood (Simpson et al., 2015). Some have reported that gold nanoparticles injected into the bloodstream in rare cases can be observed on the abluminal membrane of the BCECs, suggesting the transcytosis mechanism to be possible, but also very rare (Cabezón *et al.*, 2015; 2017; Johnsen *et al.*, 2018), while others show this route to be rather efficient for entry of TfR-targeted gold nanoparticles (Clark and Davis, 2015; Wiley et al., 2013). Additional controversies exist between those reporting TfR antibodies to pass the BBB, and those reporting the same antibodies to remain confined in the capillary wall (Friden et al., 1991; Moos and Morgan, 2001; Pardridge et al., 1991), but recent advances in antibody technology indicate that the route could indeed be feasible (Bien-Ly et al., 2014; Niewoehner et al., 2014; Sade et al., 2014; Yu et al., 2014; 2011). Although promising, the safety profile of these newer constructs is detrimental to further clinical development, turning some towards looking for new targets on the BBB for drug transport (Couch et al., 2013; Zuchero et al., 2016). With regards to the transport of nanocarriers into the brain to deliver a therapeutically active molecule (e.g. plasmid DNA), some report that expression of the encapsulated gene product can be detected in most areas of the brain after intravenous injection, hereby claiming that the whole nanoparticle and cargo is effectively transcytosed through the BBB (Shi et al., 2001b; Shi and Pardridge, 2000). Others, however, claim that the route of entry might as well have been the choroid plexus, which is also rich in TfR expression (Lichota et al., 2010). It could also be that the nanoparticle was processed in the BCEC yielding the cargo DNA product in its free form to be exported into the brain parenchyma.

There is still no consensus both with respect to the transcytosis and drug delivery potential of the TfR. Nevertheless, the evidence presented in the recent years on how to improve the technology by incorporating important aspects of the receptor sorting into the drug construct/nanocarrier design has made very encouraging contributions to the field, which hopefully will spark both further drug development and basic research in the receptor (these improvements will be discussed later in this review).

#### 4.2. TfR-targeted brain drug delivery in disorders with loss of blood-brain barrier integrity

An important aspect to take into consideration when designing drug delivery system like those targeting the TfR is whether such active targeting of the drug provides any additional benefit compared to a non-targeted variant. Also, will the disease process and the potential concomitant disruption of the BBB be so severe that the drug will accumulate in the brain regardless of a targeting molecule? The choice of using the TfR as a targeting molecule is mainly due to its exclusive expression on the surface of the BBB, but this does not necessarily work as an argument for using active targeting in diseases with increased vascular permeability (Park, 2017). TfRs are also expressed by some cell types of the brain parenchyma (e.g. neurons), and therefore, using the TfR as a target may facilitate accumulation of the drug or nanocarriers in the area of disease if the BBB integrity is disrupted (Moos, 1996; Moos et al., 2007; Skjørringe et al., 2015). Many diseases of the brain have been characterized with a component of BBB disruption as a part of the pathophysiological process (Montagne et al., 2015; Turjeman et al., 2015; Zlokovic, 2008). This leads to leakage of blood constituents or injected probes in the areas of disease, such as a site in the brain with ongoing neuroinflammation, and these molecules will likely diffuse in the brain extracellular space as a function of its size (Carvey et al., 2009; 2005; Desai Bradaric et al., 2012; Desai et al., 2007; 2009; A. Patel et al., 2011). The impact of these neurodegeneration-related BBB disruptions on brain drug delivery has only been characterized systematically in a few studies (Avnir et al., 2011; Bien-Ly et al., 2015; Kizelsztejn et al., 2009; Schmidt et al., 2003; Turjeman et al., 2015). For example, active targeting (with ApoE and  $\beta$ -amyloid peptides) on liposomes were shown not to confer additional therapeutic effects after injection into mice suffering from the induced model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), suggesting a severe breakdown of the BBB integrity in this model, since the liposomes could easily accumulate in the area of disease (Avnir et al., 2011; Kizelsztejn et al., 2009; Schmidt et al., 2003; Turjeman et al., 2015). However, this model is in many ways extreme compared to the evidence on neuroinflammatory disorders in other models and in human disease, and hence, others have used this model as a positive control for BBB disruption (Bien-Ly et al., 2015). In line with this, recent evidence indicates that the widely accepted fact of widespread BBB disruption in neurodegenerative disorders must be challenged (Alata et al., 2015; Bien-Ly et al., 2015; Z. Cheng et al., 2010; Do et al., 2014; Minh Do et al., 2015; St-Amour et al., 2013). The results of one study showed that the size-related cut-off on any potential BBB disruption was below 3 kDa, and that only antibodies (150 kDa) targeting the TfR with low affinity, as compared to non-targeting antibodies, could pass into the brain in several different preclinical models of Alzheimer's disease (Bien-Ly et al., 2015). Others showed no additional transport of a panel of small molecule drugs (266 – 720 Da, varying transport mechanisms) in preclinical models of both multiple sclerosis and Alzheimer's disease (Z. Cheng et al., 2010). However, if existing, BBB disruption may in fact be detrimental to drug delivery, because the structural changes in the NVU and the associated aberrations in perivascular and brain extracellular fluid dynamics may halt the diffusion of drugs from the capillary to the brain parenchyma (Sweeney et al., 2018). Thus, there is a strong argument for the continuous development of BBB-penetrating drug delivery strategies.

Another important questions in relation to the possible disease-associated changes to the integrity of the BBB, is whether or not the TfR expression is modulated in disease states. In the 1990s, several groups showed changes in the expression of the TfR both in Parkinson's (Faucheux et al., 1997; 1993; C. M. Morris et al., 1994b) or Alzheimer's disease (Kalaria et al., 1992; C. M. Morris et al., 1994a) using autoradiography on post-mortem human brain specimens. While these estimations often came with large regional difference, e.g. between the hippocampus and frontal cortex in Alzheimer's disease, they were rarely reflected at the capillary level (Kalaria et al., 1992), suggesting the TfR expression to be maintained in this compartment in neurodegenerative diseases. Recent studies have validated these findings by quantifying the TfR protein content of whole brain and capillary samples derived from transgenic mice or patients suffering from Alzheimer's disease (Bien-Ly et al., 2015; Bourassa et al., 2019). Others found the TfR to be at least partially functional after several hours of brain ischemia (Hao and Bickel, 2013). Although some questions related to the expression of TfR in disease states still needs to be answered, our current knowledge suggest that it remains expressed and is readily available for targeting and transporting drugs to the brain parenchyma.

#### 4.3. Challenges in addition to transcytosis of the blood-brain barrier

After having traversed the BBB, the transcytosed drug construct or nanocarrier needs to be transported to the area of disease, which would likely be neurons in the tissue surrounding the capillary. Circumventing the BBB by forcing it open can allow for the transport of nanoparticles into the brain parenchyma and can thus function as a model for what will happen after transcytosis (Åslund et al., 2015; Muldoon et al., 1999). The results of such studies are, however, very discouraging. For example, nanoparticles in size ranges suitable for high drug encapsulation (approximately 100 nm) seems to become stuck in the protein meshwork of the basement membrane located beneath the BCECs (Muldoon *et al.*, 1999; Cabezón *et al.*, 2015). The pore sizes of this basement membrane are much smaller than what would be needed for a nanoparticle to move into proximity of diseased neurons in the brain parenchyma, thus questioning the idea that whole nanoparticles can be transported into the brain parenchyma in this manner. Moreover, using pharmacokinetic modelling based on in vivo data, the *Hammarlund-Udenaes* group has proposed that the most likely outcome between a liposome and the BBB would be fusion with the endothelial membrane. This would in effect release an encapsulated cargo into the BCEC cytoplasm, wherefrom it could possibly be redistributed to the brain parenchyma for therapeutic action (Lindqvist et al., 2016). This will of course not be the case for non-lipid nanoparticles, but it does shed light on the possibility that whole nanoparticle transport across the BBB is not to be expected (Freskgård and Urich, 2017; Gosk et al., 2004), even though this is claimed in several studies (Zensi *et al.*, 2009; 2010; Wiley *et al.*, 2013; Clark and Davis, 2015; Johnsen *et al.*, 2018). The lipid composition of liposomes may also affect the cargo uptake, suggesting that unsaturated liposomes are more likely to release the encapsulated cargo to be transported into the brain extracellular space (Hu et al., 2017), whereas in some cases, liposomal encapsulation may be detrimental to the brain uptake (Hu et al., 2018). Target occupation

time and saturation of the encapsulated drug towards its therapeutic target is also of great importance when determining the effect of a liposomal brain drug delivery system (de Witte et al., 2018). Furthermore, there is disagreement as to the possible size of the brain extracellular space, and whether that would allow for movement of nanoparticles (Nance et al., 2012; Thorne and Nicholson, 2006). While most agree that proteins including antibodies will be able to move freely in the brain extracellular space (Pizzo et al., 2018; Plog et al., 2018), some measurements suggest this space to be between 38 – 64 nm (Thorne and Nicholson, 2006), which is well below the size of most nanoparticles used for drug delivery (Johnsen and Moos, 2016). Therefore, based on these results, a nanoparticle strategy for brain drug delivery would not be adequate for having the drug compound widely distributed in the brain parenchyma (Thorne and Nicholson, 2006; Wolak et al., 2015; Wolak and Thorne, 2013). Conversely, others have found that a large fraction of the brain extracellular space has pore sizes above 100 nm, which would allow for nanoparticles to distribute, although this distribution is highly affected by the amount of surface coating (PEGylation) of the nanoparticles (Nance et al., 2014b; 2012; Suk et al., 2016). This was also the case after forced opening of the BBB (Nance et al., 2014a; C. Zhang et al., 2017a; 2017b). Yet others have proposed that albumin nanoparticles of more than 200 nm in diameter can be efficiently transcytosed (although not as a result of TfR targeting) and distributed in the astrocytic processes to neurons distant from the capillary (Begley, 2012; Zensi et al., 2010; 2009).

A related challenge is also facing the TfR-targeted antibodies and especially nanoparticles even before they have been taken up into the BCEC, namely the impact of the glycocalyx (Hempel et al., 2016; Kutuzov et al., 2018). The glycocalyx is a carbohydrate-rich matrix lining the luminal surface of endothelial cells, which gives both structural support to maintain the integrity of the endothelial cell lining, and contributes to the net negative charge of the endothelial surface (Hempel et al., 2016; Santos et al., 1995). Due to the negative charge of the glycocalyx, and the fact that it spaces the cell membrane and drug construct or nanoparticle with 0.5 – 11  $\mu\text{m}$ , it has been hypothesized to impact greatly on the uptake of nanoparticles into the brain (Gromnicova et al., 2016a). This would in effect mean that the TfRs on the BCEC surface are buried in the proteoglycan matrix, and that binding to it will be dependent on the size of the construct. In line with this, the glycocalyx was recently found to be a significant diffusion barrier even to low molecular weight fluorophores (Kutuzov et al., 2018). In rat fat pad endothelial cells, PEGylated gold nanoparticles had an overall low uptake under normal conditions, but this uptake was increased markedly as a response to modulations of the glycocalyx including collapsing and enzymatic degradation (M. J. Cheng et al., 2016). This suggested that negatively charged nanoparticles have a restricted interaction with the endothelial cell (and subsequent low uptake) due to the repulsive force of the negatively charged glycocalyx (M. J. Cheng et al., 2016). Gold nanoparticles with a positively charged (aminated) PEG layer interact readily with the cell surface of endothelial cells from either kidney or brain (Gromnicova et al., 2016a). Enzymatic degradation of the glycocalyx did, however, not change the uptake of these positively charged nanoparticles into BCECs significantly (Gromnicova et al., 2016a). Conversely, the uptake of both carboxylated and aminated polystyrene nanoparticles into human umbilical

vein endothelial cells was significantly improved after enzymatic degradation of the glycocalyx, suggesting that the glycocalyx constitutes a significant barrier for nanoparticle uptake into endothelial cells in general (Möckl et al., 2017). At the moment, no data exist to predict how the glycocalyx may interfere with the association between TfR-targeted nanoparticles and the BCEC surface, although inspiration may be taken from computer simulations of the interaction between intercellular adhesion molecule 1 (ICAM-1) antibody-functionalized PLGA nanoparticles and vascular endothelium (J. Liu et al., 2010). These investigations indicate that the glycocalyx reduces the binding affinity of the antibody-functionalized nanoparticles (J. Liu et al., 2010), which may also be expected for TfR-targeted nanoparticles interacting with the brain endothelium. It thus seems that the impact of the glycocalyx is an important aspect to consider when designing TfR-targeted medicines and predicting their interaction with the surface of the BCECs.

When the TfR-targeted drug has reached the brain parenchyma, its retention in this compartment is directly related to its binding properties to intraparenchymal targets. This can be investigated by creating bispecific antibodies targeting both the TfR and another protein either related to a specific cell type or an ongoing disease (Sehlin et al., 2016). For example, researchers from Uppsala University created different formats of a bispecific antibody capable of targeting both the TfR and  $\beta$ -amyloid and showed long antibody retention in brains of transgenic mice that carried large loads of  $\beta$ -amyloid plaques, but no antibody retention when the same bispecific antibody was administered to wild-type mice or transgenic mice with  $\alpha$ -synuclein aggregates (Hultqvist et al., 2017; Sehlin et al., 2016; Syvänen et al., 2017). With proper intraparenchymal targets, the half-life of antibodies in the brain is thought to be more than a month (Bard et al., 2012; St-Amour et al., 2013). Others have suggested that having a intraparenchymal target is more important than the TfR-mediated transport across the BBB, since brain retention could be achieved over several weeks after treatment with an antibody targeting the myelin oligodendrocyte glycoprotein (MOG) (Nakano et al., 2019). It thus seems to be of great importance to be clear about the aim of any future brain drug delivery study, since only evaluating the transport module (the anti-TfR part) will be highly dependent on the time point of analysis due to the relatively fast clearance from the parenchymal compartment when retention cannot be ensured by an intraparenchymal target, whereas the ability to target a protein present in the brain parenchyma will change this rate of clearance (and therefore also the measured transport efficiency across the BBB) significantly (Bard et al., 2012; Hultqvist et al., 2017; Sehlin et al., 2016; Syvänen et al., 2017). It should be noted, however, that neurons express the TfR to some extent, although the levels may be too low to function as an effective intraparenchymal target to ensure long-term retention (Moos, 1996; Moos et al., 2007). Furthermore, some suggest that human antibodies (thought to be non-targeted) are able to accumulate passively in the murine brain over time, leading to the build-up of therapeutically relevant concentrations of the antibodies in the brain parenchyma (St-Amour et al., 2013).

#### *4.4. Biodistribution properties and potential side effects*

In recent years, there has been an increasing focus on the biodistribution, off-target accumulation, and the potential side effects that treatment with TfR-targeted medicines may include. Biodistribution analyses are mostly performed for studies of TfR-targeted nanomedicines, although with varying findings. For example, one group found very little uptake of TfR-targeted nanoparticles in peripheral organs compared to the brain (Sharma et al., 2014), whereas other groups found that most of the injected nanoparticle dose would be distributed to peripheral organs rather than to the brain (Hatakeyama et al., 2004; Johnsen et al., 2019; 2018; 2017). The TfR is highly expressed both in the liver (hepatocytes and Kupffer cells)(J. P. Lu et al., 1989; Malik et al., 2011; Naz et al., 2012; R. Singh et al., 2016), and in the spleen (red pulp and residing macrophages)(Hunt et al., 1998; J. P. Lu et al., 1989; Naz et al., 2012; R. Singh et al., 2016). This suggests that these two organs would be a major accumulation site for TfR-targeted nanomedicines, but this has not unequivocally been proven. While the liver is a major accumulation site for nanomedicines in general, there seems to be no correlation with the TfR-targeting capabilities of the given formulation (Johnsen et al., 2019; 2018; 2017). Conversely, newer findings suggest that the splenic accumulation does increase with increased targeting capabilities towards the TfR (Johnsen et al., 2018). Whether this has any negative impact on organ function has not yet been assessed. Another important note to stress is the differences observed in the biodistribution profiles between different commonly used mouse strains, which could potentially have an impact on the resulting brain uptake of TfR-targeted CNS drugs as well. Such a phenomenon was recently observed for brain uptake of adeno-associated viruses (AAVs), where transduction of the brain tissue was observed with high efficiency in C57BL/6J mice, but was completely missing in Balb/cJ mice (Challis et al., 2019). The brain expression of TfRs in rodents is also known to vary significantly from early postnatal development (Siddappa et al., 2002) to adulthood (Moos et al., 2006; 1998) and old age (L.-N. Lu et al., 2017), and the Tf binding capacity also changes with age (Moos et al., 2006; 1998). Furthermore, the level of expression of TfR expression varies in different brain regions (Hill et al., 1985; Mash et al., 1990; C. M. Morris et al., 1989). This means that the choice of animal model may significantly impact the outcome of a drug delivery study both with respect to general biodistribution and regional uptake in the brain. Recently, drug tissue binding (not TfR-related) in the brain was also found to vary significantly in individual AD patients and matched controls, even between different regions of the brain (Gustafsson et al., 2019), which suggest that for TfR-targeting medicines to survive through clinical development, the initial efficiency must be very high.

Studies on the general accumulation of TfR-targeted antibody-based medicines are not present to the same extent. Instead several groups employing TfR-targeting for antibody delivery to the brain have shown that treatment with anti-TfR antibodies may induce severe side effects. *Couch et al.* found that anti-TfR antibodies in addition to targeting TfRs on the surface of BCECs also targeted circulating reticulocytes (Couch et al., 2013; R'zik et al., 2001). This targeting led to reticulocyte destruction and acute clinical symptoms in the treated animals that were indicative of severe hemolysis (Couch et al., 2013). Interestingly, an older study reported a case of acquired iron-deficiency anemia caused by the production of auto-antibodies that

downregulated the expression of TfRs on the surface of erythrocyte progenitors, indicating that the potential side effects could be expected in humans as well (Larrick and Hyman, 1984). Complete remission of clinical and hematological symptoms was observed in the patient after immunosuppressive therapy (Larrick and Hyman, 1984). The side effects observed in mice after treatment with anti-TfR antibodies were induced by the retained effector function of the antibody's Fc domain (Couch et al., 2013). By reducing the affinity of the Fc domain to the Fc $\gamma$  receptor (by a mutation resulting in lack of glycosylation in the Fc domain), the same effects could not be elicited due to inability of inducing antibody-dependent cell-mediated cytotoxicity (ADCC), hereby significantly reducing the impact on the treated animals (Couch et al., 2013). Other mutations inhibiting the effector function, e.g. LALA-PG, was used by several groups to obtain the same effect (Lo et al., 2017; Weber et al., 2018a; 2018b). Recently, the N292G mutation was tested for reduction of effector function in a fusion construct between an anti-TfR antibody and EPO. While the effector function was successfully suppressed by the mutation, the plasma clearance was significantly higher compared to the wild-type protein after several types of administration, which was suggested to have detrimental effects on the general brain uptake and therapeutic efficacy (J. Sun et al., 2019). Parts of the reaction to anti-TfR antibodies with retained effector function was ascribed to complement-dependent pathways (Couch et al., 2013; Lo et al., 2017). Others found that full effector functionality of the antibody Fc domain was necessary for therapeutic efficacy against  $\beta$ -amyloid plaques, but that this effector function also resulted in first infusion reactions in the treated animals (Weber et al., 2018a). Neutrophils were found to be a key player in the induction of such reactions (Weber et al., 2018b). Interestingly, these infusion reactions could be diminished by including the TfR-targeting function in the form of brain shuttle modules attached to the C-terminal of the therapeutic antibody. Proper effector function was then maintained for therapeutic efficacy within the brain, whereas it was lost when the construct's anti-TfR brain shuttle modules bound circulating reticulocytes. This was hypothesized to be due to steric hindrance creating a stealth mode for effector function related to the TfR-binding (Weber et al., 2018a). Others disagree on the mere existence of systemic side effects after treatment with anti-TfR antibodies based on chronic studies with serial dosing over long periods of time (Pardridge, 2014; Y.-F. Zhang et al., 2003; Zhou et al., 2011b). However, these findings only applied when high affinity constructs were dosed in the 1 – 2 mg/kg range (Y.-F. Zhang et al., 2003; Zhou et al., 2011b), whereas a recent study showed that doses of 10 – 30 mg/kg led to a significant reduction of circulating reticulocytes and a subsequent low hematocrit in the Rhesus monkey (Pardridge et al., 2018b). In addition to these effects, micro- and astrogliosis was observed in the occipital cortex of the Rhesus monkeys, and mild to moderate axonal degeneration was observed in the sciatic nerve (Pardridge et al., 2018b). Both effects were discussed as being caused by the retained effector function of the anti-TfR fusion constructs (Pardridge et al., 2018b), which is in line with recent findings on the importance of orientation of the Fc domain (Weber et al., 2018a). Conversely, anti-human insulin receptor antibody-IDUA fusion constructs could be dosed weekly at 30 mg/kg in the Rhesus monkey without central or systemic side effects (Boado et al., 2016; 2013; 2014), and were recently tested in patients suffering from

MPSI (Giugliani et al., 2018; Pardridge et al., 2018a). Production of anti-drug antibodies (ADA) is increasingly being highlighted as a potential detrimental adverse effect of treatment with biological drugs, e.g. bispecific antibodies (Labrijn et al., 2019). This is, for example, associated with a significantly decreased survival after treatment with ipilimumab in patients suffering from metastatic melanoma (Kverneland et al., 2018), and thus, remain a great concern for the authorities when new biological drugs are being approved for clinical use (Boyman et al., 2014; Shankar et al., 2007). In the field of brain drug delivery, very little effort has been done to investigate, whether this is a similar problem for anti-TfR antibodies, although a recent study in mice revealed some increases in ADA titer after treatment with a chimeric anti-TfR antibody with or without fusion to SGSH (Boado et al., 2018). Therefore, future studies should investigate ADA production after treatment with anti-TfR antibodies to clarify whether this is a severe issue that needs to be handled.

With respect to TfR-targeted nanocarriers, very little has been reported on possible side effects. For example, liposomes functionalized with Tf proteins did not induce hemolysis *in vitro* (Sharma et al., 2014; 2013; 2012), whereas intravenous injection of antibody-functionalized liposomes did not result in any acute clinical symptoms in the treated animals (Markoutsas et al., 2014; Salvati et al., 2013; van Rooy et al., 2011b). Complete avoidance of side effects may, however, be dependent on the strength of coupling between the liposomes and antibodies. A recent study found that while antibody-functionalized gold nanoparticles could be administered to animals without side effects, liposomes with similar antibody density elicited severe infusion reactions (Johnsen et al., 2019). This effect was hypothesized to be caused by the antibodies leaving the liposome membranes since these were attached hereto using the so-called post-insertion technique (Allen et al., 2002; Johnsen et al., 2019). Such labile characteristics are also known for other lipid-anchored molecules such as fluorophores (Ladokhin et al., 1995; Münter et al., 2018; Snipstad et al., 2016). Thus, nanocarrier-associated anti-TfR antibodies may in fact be devoid of any immunostimulatory capacity, as is also known for tumor drug delivery with nanocarriers (Ahmed et al., 2015), suggesting them to be of relevance for future therapies. Furthermore, with the current knowledge on modifying the effector function of therapeutic antibody targeted to the brain via TfRs, such therapies also does not lack translational potential (Couch et al., 2013; Weber et al., 2018a).

## **5. Modifications to existing TfR drug delivery strategies to improve brain uptake**

Much of the existing data on the use of TfR-targeting as a means for brain drug delivery suggest the concept to be relevant. Nevertheless, the absolute amount of drug that is transported via this route remains very small. This issue needs to be handled for the TfR-targeted drugs to reach the clinical stages of drug development. Fortunately, the recent years have seen numerous examples of how to improve the current system to increase drug accumulation in the brain parenchyma (for modifications of nanocarriers, see Figure 4). This has in particular been the case for antibody-based medicines, where drug exposure to the brain has been increased significantly via protein engineering of the binding mode (Figure 5). These changes to the binding mode in

terms of affinity, avidity, or pH-sensitivity are not mutually exclusive, nor can we at this point decide upon one superior strategy to improve the transport of TfR-targeted antibodies. In the next section, these different improvements will be summarized.

### 5.1. Affinity

For many years, it was a broadly accepted fact in the field of TfR-mediated brain drug delivery that to secure the most efficient targeting, uptake and transcytosis, the ligand needed to bind to the target with high affinity. This was especially evident given the large number of studies published on the utility of high affinity antibodies against the rat and mouse TfR, e.g. clones OX26, 8D3, and RI7 (Pardridge, 2014). To date, these ligands have been used in more than 100 studies on brain drug delivery. Use of high affinity antibodies against the TfR was shown to mediate effective transfer of drugs to the CNS (Pardridge, 2014), but studies rarely included a thorough analysis of the transport of the ligand or nanocarriers itself, and quantification of drug uptake in the brain was often based on measurements on whole brain homogenates. By investigating the localization of the ligand and nanocarriers both via morphological assessment and tracer quantification after brain capillary depletion, it was instead found that the high affinity ligands did indeed reach the BCECs, but no evidence could suggest that they effectively transcytosed across the BBB in vivo (Alata et al., 2014; Gosk et al., 2004; Moos and Morgan, 2001; Paris-Robidas et al., 2011). It therefore seemed that a strong binding modus does not necessarily predict successful transport across the BBB.

With these findings in mind, the biotech company Genentech set out to investigate the issue on lack of transcytosis even though the BCECs were effectively targeted via the TfR. In a now highly cited paper, the Genentech group presented evidence that by sequential reduction of the antibody affinity against the TfR (i.e. several antibody clones), the transcytotic capacity improved with the amount of antibody within the brain parenchyma increasing from 0.2 – 0.6 %ID/g. Through morphological analyses it was also shown that while the high affinity variant of the antibody mainly was localized within the brain microvasculature, the low affinity variant exhibited a broad and pronounced distribution in the parenchyma of the cerebral cortex, hereby suggesting the antibodies to reach areas of potential disease processes (Figure 5)(Yu et al., 2011). While most evidence does not support any real transcytosis of the TfR itself, it was still suggested that the TfR could facilitate transcytosis of a given molecule, possibly via dissociation from the receptor in the endosomal compartment (Dennis and Watts, 2012; Yu et al., 2011). Subsequent studies of the intracellular sorting of the antibodies in the brain cortex showed a dramatic decrease in the expression level of the TfR (more than 50 % reduction) after administration of the high affinity variant antibodies (Bien-Ly et al., 2014). This suggested that upon treatment with this variant, the antibody and receptor was so tightly associated that the subsequent intracellular sorting directed the complex towards degradation. This was validated by strong co-localization between the high affinity variant and lysosomes. Conversely, there was very little change in the expression of the TfR after administration of the low affinity variant, which also correlated with a low degree of co-

localization with lysosomes. Like the observations of the original study, the low affinity variant was instead found to accumulate in the brain parenchyma (Bien-Ly et al., 2014; Yu et al., 2011). Another group has recently validated the beneficial effects of reducing the binding affinity to the TfR by engineering well-known anti-TfR antibodies (OX26 (mouse anti-rat) and 8D3 (rat anti-mouse))(Haqqani et al., 2018; Thom et al., 2018; Webster et al., 2017). These efforts resulted in improved transport across the BBB as well as therapeutic efficacy in pain models (Thom et al., 2018; Webster et al., 2017). Others insist that the affinity must be high to obtain efficient transport (Pardridge et al., 2018b). In addition, the concept of reducing the affinity to gain an increase in transcytosis does not only apply for the TfR, but was also shown for CD98hc (Zuchero et al., 2016). The concept of reducing the binding affinity was also tested in non-human primates, showing more modest results compared to the original murine studies. Therefore, the authors suggested that the affinity needs to be finely balanced in order to firstly reach optimal BCEC uptake and secondly transcytose across the BBB with no TfR degradation (Yu et al., 2014). Another group validated these findings based on a panel of commercially available antibodies against the TfR in a pulse-chase in vitro setup of BBB passage (Sade et al., 2014). The concept is still not elucidated to the same extent for larger nanocarriers (Figure 4), however, one study showed that the antibody with the highest affinity of those tested mediated the greatest level of transcytosis (Gregori et al., 2016). Conversely, using the antibodies originally developed by Genentech, it was shown that the low affinity variant anti-TfR antibody functionalized to gold nanoparticles yielded modest increases in the transport across the BBB both in vitro and in vivo (Johnsen et al., 2018).

Despite having been challenged by the findings of low affinity TfR-mediated transport for efficient brain drug delivery, support of the high affinity strategy has not diminished completely. In fact, a range of studies published recently support the notion that high affinity (and bivalent) binding is crucial for therapeutic efficacy in the brain parenchyma. IDUA, IDS, and SGSH enzymes were delivered to the brain via TfR-mediated transport fused to a chimeric antibody and could mediate therapeutic effects in models of MPSI, MPSII, and MPSIIIA, respectively (Boado et al., 2018; 2011; Zhou et al., 2012). Fusion of the chimeric antibody to TNF decoy receptors or EPO led therapeutic efficacy in models of stroke and Alzheimer's disease (Chang et al., 2018; 2017; Sumbria et al., 2013a). The common denominator of these different constructs was a very high affinity of  $K_D < 1$  nM against the TfR and the fact that they were dosed at 1 – 3 mg/kg. Conversely, the most efficacious low affinity variants (explained above) had affinities in the range of  $IC_{50} = 111 – 588$  nM (Bien-Ly et al., 2014; Couch et al., 2013; Yu et al., 2011) or  $K_D = 76 – 130$  nM (Thom et al., 2018; Webster et al., 2017) and was dosed at 20 – 30 mg/kg for therapeutic effect. Several factors can interfere with the effective transport, as illustrated by the fact that a  $K_D = 270$  nM resulted in the most transport in a human TfR knock-in mouse, whereas a  $K_D = 37$  nM was most efficient in the cynomolgus monkeys (Yu et al., 2014). The large difference both with respect to affinity and especially dosing is one of the main points of critique made against the low affinity hypothesis (Pardridge, 2014). Some suggest based on pharmacokinetic theory that the uptake properties of high affinity antibodies are selectively masked by the very high doses used to show the

improved uptake of low affinity antibodies. The TfR system will therefore become saturated when treated with high affinity antibodies, because the resulting plasma concentration will be many-fold higher than the  $K_D$  of the antibodies, whereas this is not the case for the low affinity antibodies (Chang et al., 2018; Pardridge, 2014; Yu et al., 2011). Furthermore, there is a significant difference in the area under the plasma concentration curve, because the low affinity antibodies are not cleared to the same extent as those with high affinity, which combined with the large dose will increase the risk of off-target effects (Pardridge, 2014). The extent of off-target effects was claimed to be less of a problem when treating with high affinity antibodies, since these can be dosed at approximately 1 mg/kg (Boado et al., 2018; 2011; Zhou et al., 2012). Furthermore, studies of high affinity anti-TfR fusion constructs in monkeys, as well as their recent introduction in clinical trials, underscores the fact that this strategy cannot be neglected for future TfR-mediated drug delivery to the brain (Okuyama et al., 2019; Pardridge et al., 2018b).

## 5.2. Avidity

Another approach of changing the binding mode between a ligand and the TfR is to modify the avidity of the antibody (Figure 5). Antibodies are characterized by two homologous arms containing a complementarity-determining region (antigen-binding fragments, Fab) that decides the specificity of the antibody (Cohen and Milstein, 1967). A hinge region between the Fab fragments and the Fc domain determines the flexibility of the Fab fragments with respect to binding two antigens with a certain distance in between (Reth, 2013; Shaw et al., 2019). With optimal spacing between two antigens both antibody Fab fragments can bind to their epitopes, hereby creating an avidity effects, which is important for the overall affinity of the binding and the subsequent initiation of effector functions in immune cells (Shaw et al., 2019). For many years it has been known that upon engaging of the TfR with an antibody, the valency of the antibody molecule affects the subsequent expression and intracellular movement of the TfR (Crépin et al., 2010; Lesley et al., 1989). Whereas monovalent antibodies does not seem to affect neither surface expression nor degradation of the TfR, the opposite is true when a bivalent antibody molecule interacts with the receptor (Lesley et al., 1989). Probably, the reason for these findings is due to the dimerization or multimerization of TfRs mediated by the bivalent nature of an antibody, since it does not mimic the normal engagement of Tf with its receptor (Freskgård and Urich, 2017). The essence of these findings was tested by researchers from Roche in a brain drug delivery setting (Niewoehner et al., 2014). Two antibody constructs ('brain shuttles') were produced with either one or two Fab fragments (sFab and dFab, respectively), and tested for their capabilities of transcytosis. Since the sFab construct mimicked the monovalent binding of the endogenous ligand, it was found to transcytose efficiently across the BBB, even though it associated with the receptor protein at an epitope distant to that binding endogenous Tf (Niewoehner et al., 2014). Like the antibody variant with low affinity, this monovalent sFab construct did not co-localize to any great extent with lysosomes. Conversely, administration of the dFab constructs was characterized by a much faster degree of internalization into the BCECs, but the subsequent

intracellular sorting directed the ligand-receptor complex for degradation in lysosomes rather than for transcytosis. With regards to the surface expression of the TfR, no change was found after treatment with the sFab constructs, and upon treatment with Bafilomycin A1, the sFab fragments accumulated inside the BCECs, hereby showing that the expression level and recycling properties were retained for the TfR (Niewoehner et al., 2014). Treatment with dFab resulted in a reduction of TfR surface expression to an almost undetectable level, which suggests that bivalent engagement of the TfR of BCECs severely distorts the receptor function and sorting, and may therefore not be able to mediate any efficient uptake into the brain parenchyma (Freskgård and Urich, 2017; Freskgård et al., 2014; Niewoehner et al., 2014). Supporting these findings, when the low affinity TfR (see above) and CD98hc antibodies were converted to bi-specific antibodies also targeting the BACE1 enzyme, the change in avidity greatly affected the performance of the resulting constructs (Yu et al., 2011; Zuchero et al., 2016). Other designs of monovalent binding brain shuttles were also recently described to transport an antibody-based  $\beta$ -amyloid PET tracer into the brain parenchyma (Fang et al., 2019; Hultqvist et al., 2017; Meier et al., 2018; Sehlin et al., 2017; Syvänen et al., 2018), therapeutic peptides (Ruderisch et al., 2017), or therapeutic antibodies and enzymes (Denali Therapeutics, 2018; Syvänen et al., 2018). The TfR-binding brain shuttles also served as important tools in the investigations of the pericyte influence on transendothelial transport and the presence of sorting tubules in BCECs (Villaseñor et al., 2016; 2017), whereas so-called dual variable domain and Tribody antibodies were recently explored for brain drug and PET tracer delivery (Karaoglu Hanzatian et al., 2018; Syvänen et al., 2017). In particular, the identification of sorting tubules and retrograde transporters in BCECs, their function, and their potential interplay with other intracellular organelles to mediate transcytosis will be highly important for the future development of BBB-crossing medicines (Siupka et al., 2017; Villaseñor et al., 2017; 2018). As for the affinity strategy, dosing is also of great importance when determining the value of mono- and bivalently binding anti-TfR antibodies, because the TfR system can become saturated upon administration of large quantities of the drug (Watts and Dennis, 2013; Yu et al., 2011). Trace dosing can be employed to avoid this saturation, which has yielded impressive levels of transport (2 – 3 %ID/g) after few hours (Hultqvist et al., 2017; Sumbria et al., 2013b; Watts and Dennis, 2013). However, such small doses may overestimate the efficiency of the system and the resulting level of transport cannot directly be translated to therapeutic efficacy, because this would require much larger doses (Syvänen et al., 2018; Watts and Dennis, 2013).

With respect to the delivery of large nanocarriers across the BBB, the concept of modulating the avidity to the TfR has also been tested (Figure 4) (Clark and Davis, 2015; Huwyler et al., 1996; Wiley et al., 2013). The first indications of the avidity influencing the transport properties of TfR-targeted nanoparticles came in the seminal paper by *Huwyler et al.*, who found that by increasing the ligand-to-surface ratio on PEGylated liposomes the resulting uptake into the brain changed. The increase in ligand density improved the brain uptake up to 29 antibodies per liposome, whereas higher densities (in this case 197) dramatically decreased the brain uptake (Huwyler et al., 1996). These primary observations were years later validated in a

study using gold nanoparticles functionalized with Tf on their surfaces. PEGylated gold nanoparticles of different sizes were functionalized with Tf at different surface densities and investigated for their uptake profile into the brain parenchyma (Wiley et al., 2013). As for the liposomes, an increase in surface density of Tf on the gold nanoparticles lead to higher uptake into the brain. In fact, when measuring the bulk gold content in whole brain homogenates, a surface density as high as 200 Tf molecules per 80 nm gold nanoparticles lead to a significantly higher gold content (Wiley et al., 2013). However, morphological analysis of the brain section revealed that even though high surface densities could transport large amounts of nanoparticles to the brain, most of the nanoparticles were unable to traverse the BBB, and hence they were restricted to the vessel wall (Clark and Davis, 2015; Wiley et al., 2013). Therefore, it seems that for nanoparticles to be able to pass the healthy BBB, the avidity needs to be finely tuned to reach the highest possible efficiency (Clark and Davis, 2015; Huwyler et al., 1996; Johnsen et al., 2019; Wiley et al., 2013). Too low a surface density of the ligand would likely cause fewer nanoparticles to interact with the TfR on the vessel wall (Johnsen et al., 2019), whereas too high a surface density could distort the subsequent transport of the nanoparticle across the BCEC because of simultaneous engagement of too many receptors at the time (see above)(Wiley et al., 2013). Recently, the transport capabilities of different anti-TfR antibodies functionalized to the surface of gold nanoparticles were compared (Johnsen et al., 2018; Yu et al., 2011). Following the notion that avidity is an important determinant in subsequent intracellular sorting and transport across the BBB, it was found that gold nanoparticles functionalized with a bivalently binding antibody had only little brain penetrance (Johnsen et al., 2018). This low penetrance was true even when modifying the absolute number of antibodies per nanoparticle (Johnsen et al., 2019). Conversely, gold nanoparticles functionalized with a bi-specific antibody binding the TfR monovalently were transported to the brain parenchyma with much higher efficiency, albeit still with a low absolute brain accumulation (Johnsen et al., 2018). Furthermore, these gold nanoparticles could also be detected in neural processes using electron microscopy, hereby underscoring their transcytosis potential (Johnsen et al., 2018). While this concept of tuning binding avidity of the nanoparticles still needs to be validated with more studies, some suggest that the idea of having a nanoparticle with multiple targeting ligands on its surface is severely flawed, i.e. simultaneous engagement of only a few receptors would be enough for the subsequent sorting route to be distorted, and hence, any nanoparticle that attaches to the capillary wall of the brain vasculature would not be endocytosed. If it did, it would most likely be targeted for destruction in the lysosomes (Freskgård and Urich, 2017).

### 5.3. pH

After endocytosis of the TfR, the endocytosed vesicle is sorted towards an endosomal compartment, which subsequently is acidified to induce iron release (Skjørringe et al., 2015). The iron release is thought to be driven by a reduction in the affinity of Tf to the iron after lowering the pH (Skjørringe et al., 2015). This implies that a pH-sensitive drug delivery system (Figure 5) could be a relevant strategy to employ, especially for Tf-

conjugated drugs, but it does not necessarily imply that ligands like antibodies would detach easily, since the primary incident during endosomal acidification is iron release, not protein release *per se* (Sade et al., 2014). However, iron release is not the only effect of endosomal acidification after TfR internalization. Studies of the crystal structure of the TfR have revealed that lowering of the pH leads to significant conformational changes in the receptor protein structure, which could have an impact on antibodies (or other ligands) binding to the receptor at these locations (Eckenroth et al., 2011; Lawrence et al., 1999; Steere et al., 2012).

A panel of different TfR antibodies were tested for their ability to cross an in vitro model of the BBB in a pulse-chase setup (Sade et al., 2014). While some differences in the transport potential could be explained by differences in the antibody affinity for the TfR, this characteristic could not account for all differences. Interestingly, even though two antibodies had the same binding affinity, their resulting passage properties were not equal. Instead, the observed differences were due to a difference in the pH-sensitivity of the binding mode. A pH-insensitive variant would likely show strong binding and high uptake but with a subsequent intracellular sorting directed for degradation, whereas a pH-sensitive variant would be associated with late endosomes and a higher degree of transcytosis (Sade et al., 2014). Thus, these data suggest that ligands could be modified to become activated during endosomal acidification, which would allow for subsequent intracellular sorting directed for transcytosis rather than degradation. A method to modulate the ligand to have pH-sensitivity could be to target it to areas of the TfR protein known to change its conformation during endosomal acidification, including histidine residues in the complementarity-determining region of an antibody, or to induce conformational changes in the antibody molecule itself, as was recently evidenced for heparin-sulfate proteoglycan-targeting (Eckenroth et al., 2011; Kim et al., 2016; Sade et al., 2014). Single-chain variable fragments against the TfR were also modified to become pH-sensitive, which made significant changes to the resulting intracellular sorting, although despite being interpreted in relation to BBB transport, all studies were performed in breast cancer cells (Tillotson et al., 2015). The pH-sensitivity can also be added as a design feature of nanocarriers, e.g. as a chemically reactive group between the ligand and nanoparticles (Figure 4). This construct enabled nanoparticle release from the receptor-ligand complex and induced transcytosis with subsequent distribution of the gold nanoparticles within the brain parenchyma (Clark and Davis, 2015). Recently, the same chemical linker was found to be beneficial for the delivery of mucic acid polymer nanoparticles in a model of breast cancer-derived brain metastases (Wyatt and Davis, 2019). Inclusion of the important aspect of acidification during TfR sorting in the design of targeted drug constructs or nanoparticles therefore seems very relevant. The idea could possibly be expanded to include pH-based degradation of the nanoparticle resulting in drug release inside the endothelial cell followed by drug transport into the brain parenchyma by the endothelial cell.

#### 5.4. Cell-penetrating peptides

Despite the different modifications that can be made to improve the capacity of TfR-targeting antibodies and nanocarriers, drug uptake into the brain via the TfR may only occur at very low efficiency ( $< 1\% \text{ID/g}$ ) (Johnsen and Moos, 2016). This is likely due to the physiological receptor dynamics, and therefore this uptake can be very difficult to improve substantially. One possible solution can be to include passive uptake mechanisms using cationic moieties such as lipids or cell-penetrating peptides (Figure 4). Several studies include cationic lipids in the formulation of liposomes, which is useful for encapsulation of nucleic acids, but these lipids may also have an added uptake effect due to the strong electrostatic interaction between the cationic lipid and the cell membrane (Cardoso et al., 2010; 2008; Y. Zhang et al., 2004b; 2003b; 2003a). Another approach to obtain this kind of passive uptake in the TfR-targeted system is to add cell-penetrating peptides on the surface of a nanoparticle (Sharma et al., 2014; 2013; 2012; 2016). Liposomes targeted towards the TfR using endogenous Tf were found to be superior to poly-L-arginine liposomes with respect to passing an in vitro model of the BBB. However, combining the two types of targeting improved the BBB passage significantly (Sharma et al., 2012). In vivo, the same liposomes could also effectively accumulate in the brain with high efficiency (approximately  $4\% \text{ID/g}$ ) and mediate expression of  $\beta$ -galactosidase in the brain parenchyma (Sharma et al., 2013). Furthermore, the same effects could also be obtained using other kinds of cell-penetrating peptides, most prominently for the HIV-peptide, TAT (Sharma et al., 2016; 2014). Using chitosan-based polymer nanoparticles or inclusion of other types of cationic moieties in polymer nanoparticle formulations can also facilitate this unspecific interaction between the nanocarriers and the cell membrane (Aktaş et al., 2005; Kuo et al., 2011; Yemisci et al., 2015; 2012). Additional active targeting of nanocarriers together with TfR-targeting is an interesting approach for obtaining better BBB penetrance of the carried drug. However, our understanding of the uptake mechanism has numerous gaps. For example, we do not know at which stage in the uptake process the cationic charge comes into play, i.e. whether the interaction happens already at the luminal membrane (the most likely site), or whether the charge is somehow shielded until the endocytosis into the BCEC has completed. The cationic nature of such nanoparticles may also reduce its clinical relevance in several ways, including reduced circulatory properties, unspecific uptake in other organs and toxicity, because of the chemical properties of e.g. a cationic lipid (Allen and Cullis, 2013). Still, it may be possible to optimize the design of the resulting cationic liposome to improve its clinical relevance (Heyes et al., 2005; Semple et al., 2010). From the biodistribution data presented for the liposomal formulation mentioned above, it seems that the increase in uptake observed for the brain using cell-penetrating peptides correlates with an overall higher uptake in the peripheral organs, which may be problematic with respect to the risk of side effects and low bioavailability in the blood compartment (Sharma et al., 2014; 2013).

##### *5.5. Degradation or activation of nanoparticles*

In relation to the additional targeting mediated by cationic modification of nanoparticles mentioned above, such dual targeting or drug release may also be achieved in a controlled manner by activation of the

nanoparticle at the site of interest, i.e. at the BBB (Figure 4). Such modifications are well-known for nanoparticles designed for use in non-CNS diseases such as cancer in peripheral tissues, where the pathophysiological changes in the microenvironment can trigger a destabilization of the nanoparticle to either release its drug cargo or expose an additional targeting moiety (e.g. a cell-penetrating peptide)(Gjetting *et al.*, 2014; Kaasgaard and Andresen, 2010; Torchilin, 2014). This would, however, demand that the nanoparticle would reach the area of disease for the drug to have its proper function, and as mentioned earlier, nanoparticles may not readily accumulate even though there may be some disruption of the BBB integrity (Bien-Ly *et al.*, 2015; Muldoon *et al.*, 1999). Brain cancers may be particularly relevant, although disruptions in the blood-tumor barrier may be heterogeneously distributed throughout the tumor tissue (Bruun *et al.*, 2015; Humle *et al.*, 2016; Lyle *et al.*, 2016; Steeg, 2016; Sarkaria *et al.*, 2017). In the case of brain cancer, it could also be relevant to design the nanoparticles to release its drug content in response to or propagate an external stimulus, such as ultrasound or heat stimulation (Eldridge *et al.*, 2016; Mead *et al.*, 2016; Nance *et al.*, 2014a). This is particularly the case because the current nanoparticle formulations used in the clinic do not have a high propensity to interact with and become taken up into brain tumor cells (Gudbergsson *et al.*, 2019). In addition, if the nanoparticles can transcytose through the BCEC, they will likely be restricted from moving into the neural tissue due to the basement membrane of the BBB (Muldoon *et al.*, 1999; Cabezón *et al.*, 2015). The drug would therefore need to be released from the transcytosed nanoparticle and itself diffuse towards the area of disease. Moreover, activation of the nanoparticle could be initiated inside the BCEC (Torchilin, 2014). In this way, the drug could be released either to be transported further into the brain by re-packaging by the BCEC, or function in the BCEC itself, e.g. by inducing the expression of therapeutic proteins that could be secreted into the brain parenchyma (Burkhart *et al.*, 2014; 2017; 2015; Jiang *et al.*, 2003).

### 5.6. Administration routes

To circumvent the obstacles imposed by the BBB, increasing attention has been devoted to the development of approaches to administrate the therapeutic antibodies and nanoparticles directly to the brain compartment (Woodworth *et al.*, 2014; C. Zhang *et al.*, 2017a). For example, different types of nanocarriers can be injected directly into the brain parenchyma and distribute in the extracellular or perivascular spaces using so-called convection enhanced delivery (CED), hereby covering large parts of the brain with either the nanoparticles or the carried cargo (Hadaczek *et al.*, 2006; Krauze *et al.*, 2005; MacKay *et al.*, 2005; R. Saito *et al.*, 2006), or they can be delivered to a focused area using ultrasound and microbubbles (Åslund *et al.*, 2015; Baghirov *et al.*, 2016). The gain in therapeutic efficacy was, however, not clearly observed when assessed in a brain tumor model (Sulheim *et al.*, 2019). Antibodies and nanocarriers can also be administrated via the lateral ventricles or directly into the subarachnoid space via the cisterna magna to obtain brain distribution facilitated by the flow of cerebrospinal fluid (CSF)(Liao *et al.*, 2018; A. W. J. Morris *et al.*, 2016; Pizzo *et al.*, 2018; Plog *et al.*, 2018; Samaranch *et al.*, 2013; Yadav *et al.*, 2017). Administration of TfR-targeted antibodies or nanocarriers

has not yet been attempted via these direct routes, although inspiration could be drawn to benefit the field of TfR-targeted brain drug delivery and the further transport and diffusion of TfR-targeted drugs in the brain extracellular space.

The CSF is produced in the choroid plexus, wherefrom it passes the epithelium-covered lateral and third ventricles to enter the fourth ventricle via the aqueduct of Sylvius. From the fourth ventricle, it reaches the subarachnoid space where it (among other things) acts as a buoyancy fluid that covers the brain, in addition to reaching into the brain cortex alongside penetrating vessels (i.e. the perivascular spaces)(Bakker et al., 2016). There is still a lack of agreement on matters such as terminology regarding the peri- and paravascular spaces, the direction of the CSF flow in these spaces, and the extent and types of exchange between the CSF and the brain interstitial fluid. Some argue for the so-called glymphatic hypothesis (Iliff et al., 2012; Xie et al., 2013), whereas some disagree on matters regarding the direction of the peri- and paravascular flow proposed in this model (Bakker et al., 2016; Bedussi et al., 2017). Others propose these spaces to be identical (Bedussi et al., 2017). Furthermore, the validity of the glymphatic system as a model for movement of fluid through the brain is also highly disputed (Abbott et al., 2018; Ma et al., 2019). Nevertheless, it seems clear that compounds administrated into the CSF are expected to reach the subarachnoid space and (to some degree) penetrate the brain cortex alongside the leptomeningeal arteries (Abbott et al., 2018; Pizzo et al., 2018). Although these administration routes are more invasive than intravenous injection of the antibodies or nanoparticles, they have the potential of yielding much higher local drug concentrations, which may be crucial for therapeutic efficacy (Lilius et al., 2019; Pizzo et al., 2018; Plog et al., 2018).

For example, focus has been given to the direct administration of antibodies or nanoparticles via CED into the brain parenchyma or the CSF for subsequent distribution in the respective fluid compartments (Pizzo et al., 2018; Plog et al., 2018; Woodworth et al., 2014; C. Zhang et al., 2017a). The most prominent result from several studies of CED with nanoparticles is that the distribution is mediated by transport in the perivascular compartment (Barua et al., 2012; Foley et al., 2012; Hadaczek et al., 2006; Krauze et al., 2005; 2006; Muldoon et al., 2004; Saitou et al., 2000; Salegio et al., 2014; C. Zhang et al., 2017a). In fact, the administrated nanoparticles seems to be sequestered in this perivascular space, mainly because the high resistance of the brain extracellular space favors accumulation in these less resistive compartments (Foley et al., 2012). This can in some instances be beneficial, e.g. when administering an anti-angiogenic drug in liposomes for the treatment of brain tumors, where perivascular retention increases the local drug concentration (R. Saito et al., 2006; 2004). However, in many cases, such perivascular retention will likely impede proper distribution of the nanoparticle-encapsulated drug, and hence, reduce the therapeutic efficacy of the drug delivery strategy (C. Zhang et al., 2017a). Conversely, more widespread distribution in the brain may be achieved via administration of antibodies or nanocarriers into the CSF, also known as intrathecal administration (Andersen et al., 2016; Yadav et al., 2017). Although this type of administration has been criticized for yielding very low drug concentrations of chemotherapeutic drugs (Woodworth et al., 2014), delivery of antibodies and nanocarriers

via this route has provided interesting evidence for its relevance (A. W. J. Morris et al., 2016; Pizzo et al., 2018; Plog et al., 2018; Samaranch et al., 2013). For example, three recent studies used intrathecal administration to ensure a broad brain distribution of antibodies and antibody fragments (Lilius et al., 2019; Pizzo et al., 2018; Plog et al., 2018), e.g. to bind  $\beta$ -amyloid plaques in a mouse model of Alzheimer's disease (Plog et al., 2018). The most significant effect was, however, both dependent on the size of the molecule and co-administration of hyperosmolar concentrations of mannose (Pizzo et al., 2018; Plog et al., 2018) or dexmedetomidine (Lilius et al., 2019).

Omitting the BBB completely will in principle reduce the relevance of TfR-targeting, since no transport across the barrier is needed. The most recent evidence on the transport of anti-TfR antibodies suggest that transport across the BBB can be made highly efficient (Niewoehner et al., 2014; Yu et al., 2011), and thus, the need for additional administration routes is little. However, the TfR is also an important target on cells of the brain, including neurons (Moos, 1996; Moos et al., 1998), which may illustrate a relevance of TfR-targeting after direct administration. Direct administrated TfR-targeted diphtheria toxin has previously progressed to phase III clinical trials, suggesting such administration routes to be viable and relevant (Chandramohan et al., 2017; Laske et al., 1997; Weaver and Laske, 2003).

## 6. Conclusion

Drug delivery to the brain remains a severe obstacle for treatment of neurological diseases due to the hindering imposed by the BBB. Since the passive accumulation of therapeutically active substances is almost non-existing, approaches utilizing targeting of BBB-associated receptor proteins have been heavily investigated for several decades. Amongst the different receptor systems that can be targeted, the TfR has remained the most important for brain drug delivery. The main advantages in targeting the TfR lies in its exclusive expression in brain vasculature compared to peripheral vasculature, which is thought to enable acute accumulation of therapeutically active molecules in the brain.

There is still no consensus regarding the intracellular sorting of the TfR in BCECs, especially with respect to its potential of being transcytosed. Most of the research field dedicated to the study of brain iron uptake favors a model without transcytosis, whereas the field of brain drug delivery relies on the possibility of transcytosis to explain their improved efficacy of TfR-targeted medicines compared to non-targeted variants. Therefore, it is of great importance for these two fields to converge in order to fully understand the functionality and sorting potential of the TfR. This will provide both basic knowledge on brain iron handling as well as indications on how to design efficiently transported brain drug delivery systems. The recent development of pH-sensitive drug delivery systems that take advantage of the intracellular sorting of the TfR is a good example of this.

Regardless of the lack of common grounds with respect to the fundamental understanding of the TfR, it has been used for brain drug delivery purposes for almost 30 years. The many studies have unambiguously

underscored its relevance as a brain drug delivery target, and some even suggest it to be superior to other receptor systems. This is illustrated further by the fact that the pharmaceutical and biotechnological industry has a great interest in studying the TfR. The interest from the industry has also paved the way for some significant leaps forward in our understanding of the binding mode between the TfR and a targeting ligand or antibody, and how this can depict the downstream uptake process, intracellular sorting, and transport into the brain parenchyma. As such, we now know that modulation of the binding affinity and avidity can be very beneficial for the transport of antibodies into the brain parenchyma, where they are able to elicit their therapeutic potential. Progression of these projects into clinical trials are expected very soon, and the results of these trials are expected with great anticipation. Nevertheless, the classical strategy of high affinity binding should not be neglected, since the amount of available evidence in support of this strategy is vast and the strategy has progressed into clinical trials. Successful development of clinically relevant TfR-targeted, antibody-based brain medicines will also require a larger focus on the translational aspect of interspecies differences in TfR expression and function. The recent introduction of human TfR knock-in animal models and cynomolgus monkeys by the biopharmaceutical industry in their preclinical development platforms will therefore likely inspire academic researchers to study this aspect more in depth in the future. Furthermore, additional knowledge on the impact of intraparenchymal targets on the retention of antibodies in the brain will likely lead us to design better studies in the future that can fully illustrate the potential of any given TfR-targeted, antibody-based brain medicine.

Nanomedicine has been a great focus point for drug delivery in general, and many studies indicate that TfR-targeting of different kinds of nanoparticles to the brain is a feasible strategy to increase drug exposure in the brain. However, compared to antibody-based medicines, the field of nanomedicine has not progressed as far, even though initial targeting of the BCECs is very efficient via the TfR. The reasons for this may be many-fold, but several quantitative assessments of nanoparticle and/or cargo uptake into the brain parenchyma indicates that the overall transport efficiency is at least an order of magnitude lower than that for antibody-based medicines. This is likely because of the big difference in size, and time will tell whether this efficiency can be increased to a stage relevant for clinical development. If not, there are multiple ways TfR-targeted nanomedicines could be modified in order to obtain a better drug exposure to the brain. These strategies involve the introduction of different types of sensitivities (e.g. towards redox potential or pH) or modes of activation, which would make the nanoparticle disintegrate to release its encapsulated cargo, or expose a cell-penetrating peptide that will enable further transport from the BCEC to the brain parenchyma. Inclusion of these strategies in nanomedicine will be of great importance for future studies.

The past decade has been enormously important for the field of TfR-mediated brain drug delivery, and the new inventions have sparked renewed motivation in developing this therapeutic concept further. With the expectation of further clinical progression very soon, the next years will be of great interest for the entire field of brain drug delivery.

## Acknowledgements

The most recent results obtained and described by the authors were generated by generous grants from the Lundbeck Foundation Research Initiative on Brain Barriers and Drug Delivery ([www.ribbdd.dk](http://www.ribbdd.dk))(Grant no: 2013-14113) and Fonden til Lægevidenskabens Fremme (14-191).

## References

- Abbott, N.J., 2013. Blood-brain barrier structure and function and the challenges for CNS drug delivery. *J. Inherit. Metab. Dis.* 36, 437–449. doi:10.1007/s10545-013-9608-0
- Abbott, N.J., Patabendige, A.A.K., Dolman, D.E.M., Yusof, S.R., Begley, D.J., 2010. Structure and function of the blood-brain barrier. *Neurobiol. Dis.* 37, 13–25. doi:10.1016/j.nbd.2009.07.030
- Abbott, N.J., Pizzo, M.E., Preston, J.E., Janigro, D., Thorne, R.G., 2018. The role of brain barriers in fluid movement in the CNS: is there a “glymphatic” system? *Acta Neuropathol.* 135, 387–407. doi:10.1007/s00401-018-1812-4
- Abbott, N.J., Rönnbäck, L., Hansson, E., 2006. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat. Rev. Neurosci.* 7, 41–53. doi:10.1038/nrn1824
- Agrawal, P., Singh, R.P., Sonali, Kumari, L., Sharma, G., Koch, B., Rajesh, C.V., Mehata, A.K., Singh, S., Pandey, B.L., Muthu, M.S., 2017a. TPGS-chitosan cross-linked targeted nanoparticles for effective brain cancer therapy. *Mater Sci Eng C Mater Biol Appl* 74, 167–176. doi:10.1016/j.msec.2017.02.008
- Agrawal, P., Sonali, Singh, R.P., Sharma, G., Mehata, A.K., Singh, S., Rajesh, C.V., Pandey, B.L., Koch, B., Muthu, M.S., 2017b. Bioadhesive micelles of d- $\alpha$ -tocopherol polyethylene glycol succinate 1000: Synergism of chitosan and transferrin in targeted drug delivery. *Colloids Surf B Biointerfaces* 152, 277–288. doi:10.1016/j.colsurfb.2017.01.021
- Ahmed, M., Pan, D.W., Davis, M.E., 2015. Lack of in vivo antibody dependent cellular cytotoxicity with antibody containing gold nanoparticles. *Bioconjug. Chem.* 26, 812–816. doi:10.1021/acs.bioconjchem.5b00139
- Aisen, P., Leibman, A., Zweier, J., 1978. Stoichiometric and site characteristics of the binding of iron to human transferrin. *J. Biol. Chem.* 253, 1930–1937.
- Aktaş, Y., Yemisci, M., Andrieux, K., Gürsoy, R.N., Alonso, M.J., Fernandez-Megia, E., Novoa-Carballal, R., Quiñoá, E., Riguera, R., Sargon, M.F., Celik, H.H., Demir, A.S., Hincal, A.A., Dalkara, T., Capan, Y., Couvreur, P., 2005. Development and brain delivery of chitosan-PEG nanoparticles functionalized with the monoclonal antibody OX26. *Bioconjug. Chem.* 16, 1503–1511. doi:10.1021/bc050217o
- Alata, W., Paris-Robidas, S., Emond, V., Bourasset, F., Calon, F., 2014. Brain uptake of a fluorescent vector targeting the transferrin receptor: a novel application of in situ brain perfusion. *Mol. Pharm.* 11, 243–253. doi:10.1021/mp400421a

- Alata, W., Ye, Y., St-Amour, I., Vandal, M., Calon, F., 2015. Human apolipoprotein E  $\epsilon$ 4 expression impairs cerebral vascularization and blood-brain barrier function in mice. *J. Cereb. Blood Flow Metab.* 35, 86–94. doi:10.1038/jcbfm.2014.172
- Albeck, D.S., Hoffer, B.J., Quissell, D., Sanders, L.A., Zerbe, G., Granholm, A.C., 1997. A non-invasive transport system for GDNF across the blood-brain barrier. *Neuroreport* 8, 2293–2298.
- Aldred, A.R., Dickson, P.W., Marley, P.D., Schreiber, G., 1987. Distribution of transferrin synthesis in brain and other tissues in the rat. *J. Biol. Chem.* 262, 5293–5297.
- Allen, T.M., Cullis, P.R., 2013. Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Deliv. Rev.* 65, 36–48. doi:10.1016/j.addr.2012.09.037
- Allen, T.M., Sapra, P., Moase, E., 2002. Use of the post-insertion method for the formation of ligand-coupled liposomes. *Cell. Mol. Biol. Lett.* 7, 889–894.
- Altamura, S., Muckenthaler, M.U., 2009. Iron toxicity in diseases of aging: Alzheimer's disease, Parkinson's disease and atherosclerosis. *J Alzheimers Dis* 16, 879–895. doi:10.3233/JAD-2009-1010
- Andersen, H.H., Johnsen, K.B., Arendt-Nielsen, L., 2016. On the prospect of clinical utilization of microRNAs as biomarkers or treatment of chronic pain. *Exp. Neurol.* 284, 63–66. doi:10.1016/j.expneurol.2016.07.008
- Anderson, G.J., Vulpe, C.D., 2009. Mammalian iron transport. *Cell. Mol. Life Sci.* 66, 3241–3261. doi:10.1007/s00018-009-0051-1
- Armulik, A., Genové, G., Betsholtz, C., 2011. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev. Cell* 21, 193–215. doi:10.1016/j.devcel.2011.07.001
- Armulik, A., Genové, G., Mäe, M., Nisancioglu, M.H., Wallgard, E., Niaudet, C., He, L., Norlin, J., Lindblom, P., Strittmatter, K., Johansson, B.R., Betsholtz, C., 2010. Pericytes regulate the blood-brain barrier. *Nature* 468, 557–561. doi:10.1038/nature09522
- Avnir, Y., Turjeman, K., Tulchinsky, D., Sigal, A., Kizelsztejn, P., Tzemach, D., Gabizon, A., Barenholz, Y., 2011. Fabrication principles and their contribution to the superior in vivo therapeutic efficacy of nano-liposomes remote loaded with glucocorticoids. *PLoS ONE* 6, e25721. doi:10.1371/journal.pone.0025721
- Åslund, A.K.O., Berg, S., Hak, S., Mørch, Ý., Torp, S.H., Sandvig, A., Widerøe, M., Hansen, R., de Lange Davies, C., 2015. Nanoparticle delivery to the brain — By focused ultrasound and self-assembled nanoparticle-stabilized microbubbles. *Journal of Controlled Release* 220, 287–294. doi:10.1016/j.jconrel.2015.10.047
- Baghirov, H., Karaman, D., Viitala, T., Duchanoy, A., Lou, Y.-R., Mamaeva, V., Pryazhnikov, E., Khiroug, L., de Lange Davies, C., Sahlgren, C., Rosenholm, J.M., 2016. Feasibility Study of the Permeability and Uptake of Mesoporous Silica Nanoparticles across the Blood-Brain Barrier. *PLoS ONE* 11, e0160705. doi:10.1371/journal.pone.0160705

- Bakker, E.N.T.P., Bacskai, B.J., Arbel-Ornath, M., Aldea, R., Bedussi, B., Morris, A.W.J., Weller, R.O., Carare, R.O., 2016. Lymphatic Clearance of the Brain: Perivascular, Paravascular and Significance for Neurodegenerative Diseases. *Cell. Mol. Neurobiol.* 36, 181–194. doi:10.1007/s10571-015-0273-8
- Bard, F., Fox, M., Friedrich, S., Seubert, P., Schenk, D., Kinney, G.G., Yednock, T., 2012. Sustained levels of antibodies against A $\beta$  in amyloid-rich regions of the CNS following intravenous dosing in human APP transgenic mice. *Exp. Neurol.* 238, 38–43. doi:10.1016/j.expneurol.2012.07.022
- Barua, N.U., Bienemann, A.S., Hesketh, S., Wyatt, M.J., Castrique, E., Love, S., Gill, S.S., 2012. Intrastratial convection-enhanced delivery results in widespread perivascular distribution in a pre-clinical model. *Fluids Barriers CNS* 9, 2. doi:10.1186/2045-8118-9-2
- Bäckman, C., Biddle, P.T., Ebendal, T., Friden, P.M., Gerhardt, G.A., Henry, M.A., Mackerlova, L., Söderström, S., Strömberg, I., Walus, L., 1995. Effects of transferrin receptor antibody-NGF conjugate on young and aged septal transplants in oculo. *Exp. Neurol.* 132, 1–15.
- Bäckman, C., Rose, G.M., Hoffer, B.J., Henry, M.A., Bartus, R.T., Friden, P., Granholm, A.C., 1996. Systemic administration of a nerve growth factor conjugate reverses age-related cognitive dysfunction and prevents cholinergic neuron atrophy. *J. Neurosci.* 16, 5437–5442.
- Beard, J.L., Dawson, H., Piñero, D.J., 1996. Iron metabolism: a comprehensive review. *Nutr. Rev.* 54, 295–317.
- Bedussi, B., van der Wel, N.N., de Vos, J., van Veen, H., Siebes, M., VanBavel, E., Bakker, E.N., 2017. Paravascular channels, cisterns, and the subarachnoid space in the rat brain: A single compartment with preferential pathways. *J. Cereb. Blood Flow Metab.* 37, 1374–1385. doi:10.1177/0271678X16655550
- Begley, D.J., 2012. Brain superhighways. *Sci Transl Med* 4, 147fs29. doi:10.1126/scitranslmed.3004611
- Begley, D.J., 2004. ABC transporters and the blood-brain barrier. *Curr. Pharm. Des.* 10, 1295–1312.
- Begley, D.J., Brightman, M.W., 2003. Structural and functional aspects of the blood-brain barrier. *Prog Drug Res* 61, 39–78.
- Benkovic, S.A., Connor, J.R., 1993. Ferritin, transferrin, and iron in selected regions of the adult and aged rat brain. *J. Comp. Neurol.* 338, 97–113. doi:10.1002/cne.903380108
- Bhattacharya, R., Osburg, B., Fischer, D., Bickel, U., 2008. Targeted delivery of complexes of biotin-PEG-polyethylenimine and NF-kappaB decoys to brain-derived endothelial cells in vitro. *Pharm. Res.* 25, 605–615. doi:10.1007/s11095-007-9389-y
- Bi, Y., Liu, L., Lu, Y., Sun, T., Shen, C., Chen, X., Chen, Q., An, S., He, X., Ruan, C., Wu, Y., Zhang, Y., Guo, Q., Zheng, Z., Liu, Y., Lou, M., Zhao, S., Jiang, C., 2016. T7 Peptide-Functionalized PEG-PLGA Micelles Loaded with Carmustine for Targeting Therapy of Glioma. *ACS Appl Mater Interfaces* 8, 27465–27473. doi:10.1021/acsami.6b05572
- Bickel, U., Kang, Y.S., Pardridge, W.M., 1995. In vivo cleavability of a disulfide-based chimeric opioid peptide in rat brain. *Bioconjug. Chem.* 6, 211–218.

- Bickel, U., Kang, Y.S., Yoshikawa, T., Pardridge, W.M., 1994. In vivo demonstration of subcellular localization of anti-transferrin receptor monoclonal antibody-colloidal gold conjugate in brain capillary endothelium. *J. Histochem. Cytochem.* 42, 1493–1497. doi:10.1177/42.11.7930531
- Bickel, U., Yoshikawa, T., Landaw, E.M., Faull, K.F., Pardridge, W.M., 1993. Pharmacologic effects in vivo in brain by vector-mediated peptide drug delivery. *Proc. Natl. Acad. Sci. U.S.A.* 90, 2618–2622.
- Bien-Ly, N., Boswell, C.A., Jeet, S., Beach, T.G., Hoyte, K., Luk, W., Shihadeh, V., Ulufatu, S., Foreman, O., Lu, Y., DeVoss, J., van der Brug, M., Watts, R.J., 2015. Lack of Widespread BBB Disruption in Alzheimer's Disease Models: Focus on Therapeutic Antibodies. *Neuron* 88, 289–297. doi:10.1016/j.neuron.2015.09.036
- Bien-Ly, N., Yu, Y.J., Bumbaca, D., Elstrott, J., Boswell, C.A., Zhang, Y., Luk, W., Lu, Y., Dennis, M.S., Weimer, R.M., Chung, I., Watts, R.J., 2014. Transferrin receptor (TfR) trafficking determines brain uptake of TfR antibody affinity variants. *J. Exp. Med.* 211, 233–244. doi:10.1084/jem.20131660
- Biogen, 2019. Biogen and Eisai to discontinue Phase 3 ENGAGE and EMERGE trials of aducanumab in Alzheimer's disease.
- Bloch, B., Popovici, T., Levin, M.J., Tuil, D., Kahn, A., 1985. Transferrin gene expression visualized in oligodendrocytes of the rat brain by using in situ hybridization and immunohistochemistry. *Proc. Natl. Acad. Sci. U.S.A.* 82, 6706–6710.
- Boado, R.J., Hui, E.K.-W., Lu, J.Z., Pardridge, W.M., 2016. Very High Plasma Concentrations of a Monoclonal Antibody against the Human Insulin Receptor Are Produced by Subcutaneous Injection in the Rhesus Monkey. *Mol. Pharm.* 13, 3241–3246. doi:10.1021/acs.molpharmaceut.6b00456
- Boado, R.J., Hui, E.K.-W., Lu, J.Z., Pardridge, W.M., 2013. IgG-enzyme fusion protein: pharmacokinetics and anti-drug antibody response in rhesus monkeys. *Bioconjug. Chem.* 24, 97–104. doi:10.1021/bc3005123
- Boado, R.J., Hui, E.K.-W., Lu, J.Z., Zhou, Q.-H., Pardridge, W.M., 2011. Reversal of lysosomal storage in brain of adult MPS-I mice with intravenous Trojan horse-iduronidase fusion protein. *Mol. Pharm.* 8, 1342–1350. doi:10.1021/mp200136x
- Boado, R.J., Ji, A., Pardridge, W.M., 2000. Cloning and expression in *Pichia pastoris* of a genetically engineered single chain antibody against the rat transferrin receptor. *J Drug Target* 8, 403–412. doi:10.3109/10611860008997916
- Boado, R.J., Ka-Wai Hui, E., Zhiqiang Lu, J., Pardridge, W.M., 2014. Insulin receptor antibody-iduronate 2-sulfatase fusion protein: pharmacokinetics, anti-drug antibody, and safety pharmacology in Rhesus monkeys. *Biotechnol. Bioeng.* 111, 2317–2325. doi:10.1002/bit.25289
- Boado, R.J., Lu, J.Z., Hui, E.K.-W., Pardridge, W.M., 2018. Reduction in Brain Heparan Sulfate with Systemic Administration of an IgG Trojan Horse-Sulfamidase Fusion Protein in the Mucopolysaccharidosis Type IIIA Mouse. *Mol. Pharm.* 15, 602–608. doi:10.1021/acs.molpharmaceut.7b00958

- Boado, R.J., Pardridge, W.M., 2011. The Trojan Horse Liposome Technology for Nonviral Gene Transfer across the Blood-Brain Barrier. *J Drug Deliv* 2011, 296151. doi:10.1155/2011/296151
- Boado, R.J., Zhang, Y., Wang, Y., Pardridge, W.M., 2009. Engineering and expression of a chimeric transferrin receptor monoclonal antibody for blood-brain barrier delivery in the mouse. *102*, 1251–1258. doi:10.1002/bit.22135
- Boado, R.J., Zhou, Q.-H., Lu, J.Z., Hui, E.K.-W., Pardridge, W.M., 2010. Pharmacokinetics and brain uptake of a genetically engineered bifunctional fusion antibody targeting the mouse transferrin receptor. *Mol. Pharm.* 7, 237–244. doi:10.1021/mp900235k
- Bommana, M.M., Kirthivasan, B., Squillante, E., 2012. In vivo brain microdialysis to evaluate FITC-dextran encapsulated immunopeglylated nanoparticles. *Drug Deliv* 19, 298–306. doi:10.3109/10717544.2012.714812
- Bourassa, P., Alata, W., Tremblay, C., Paris-Robidas, S., Calon, F., 2019. Transferrin receptor-mediated uptake at the blood-brain barrier is not impaired by Alzheimer's disease neuropathology. *Mol. Pharm.* acs.molpharmaceut.8b00870. doi:10.1021/acs.molpharmaceut.8b00870
- Boyman, O., Comte, D., Spertini, F., 2014. Adverse reactions to biologic agents and their medical management. *Nat Rev Rheumatol* 10, 612–627. doi:10.1038/nrrheum.2014.123
- Brightman, M.W., Kaya, M., 2000. Permeable endothelium and the interstitial space of brain. *Cell. Mol. Neurobiol.* 20, 111–130.
- Broadwell, R.D., Baker-Cairns, B.J., Friden, P.M., Oliver, C., Villegas, J.C., 1996. Transcytosis of protein through the mammalian cerebral epithelium and endothelium. III. Receptor-mediated transcytosis through the blood-brain barrier of blood-borne transferrin and antibody against the transferrin receptor. *Exp. Neurol.* 142, 47–65. doi:10.1006/exnr.1996.0178
- Burdo, J.R., Martin, J., Menzies, S.L., Dolan, K.G., Romano, M.A., Fletcher, R.J., Garrick, M.D., Garrick, L.M., Connor, J.R., 1999. Cellular distribution of iron in the brain of the Belgrade rat. *Neuroscience* 93, 1189–1196.
- Burdo, J.R., Menzies, S.L., Simpson, I.A., Garrick, L.M., Garrick, M.D., Dolan, K.G., Haile, D.J., Beard, J.L., Connor, J.R., 2001. Distribution of divalent metal transporter 1 and metal transport protein 1 in the normal and Belgrade rat. *J. Neurosci. Res.* 66, 1198–1207. doi:10.1002/jnr.1256
- Burkhart, A., Andresen, T.L., Aigner, A., Thomsen, L.B., Moos, T., 2017. Transfection of primary brain capillary endothelial cells for protein synthesis and secretion of recombinant erythropoietin: a strategy to enable protein delivery to the brain. *Cell. Mol. Life Sci.* 74, 2467–2485. doi:10.1007/s00018-017-2501-5
- Burkhart, A., Azizi, M., Thomsen, M.S., Thomsen, L.B., Moos, T., 2014. Accessing Targeted Nanoparticles to the Brain: The Vascular Route. *Curr. Med. Chem.*
- Burkhart, A., Skjørringe, T., Johnsen, K.B., Siupka, P., Thomsen, L.B., Nielsen, M.S., Thomsen, L.L., Moos, T., 2016. Expression of Iron-Related Proteins at the Neurovascular Unit Supports Reduction and

- Reoxidation of Iron for Transport Through the Blood-Brain Barrier. *Mol Neurobiol* 53, 7237–7253. doi:10.1007/s12035-015-9582-7
- Burkhart, A., Thomsen, L.B., Thomsen, M.S., Lichota, J., Fazakas, C., Krizbai, I., Moos, T., 2015. Transfection of brain capillary endothelial cells in primary culture with defined blood-brain barrier properties. *Fluids Barriers CNS* 12, 19. doi:10.1186/s12987-015-0015-9
- Cabezón, I., Augé, E., Bosch, M., Beckett, A.J., Prior, I.A., Pelegrí, C., Vilaplana, J., 2017. Serial block-face scanning electron microscopy applied to study the trafficking of 8D3-coated gold nanoparticles at the blood-brain barrier. *Histochem. Cell Biol.* 36, 437. doi:10.1007/s00418-017-1553-9
- Cabezón, I., Manich, G., Martin-Venegas, R., Camins, A., Pelegrí, C., Vilaplana, J., 2015. Trafficking of gold nanoparticles coated with the 8D3 anti-transferrin receptor antibody at the mouse blood-brain barrier. *Mol. Pharm.* 151005220742000. doi:10.1021/acs.molpharmaceut.5b00597
- Cardoso, A.L.C., Costa, P., de Almeida, L.P., Simões, S., Plesnila, N., Culmsee, C., Wagner, E., de Lima, M.C.P., 2010. Tf-lipoplex-mediated c-Jun silencing improves neuronal survival following excitotoxic damage in vivo. *J Control Release* 142, 392–403. doi:10.1016/j.jconrel.2009.11.004
- Cardoso, A.L.C., Simões, S., de Almeida, L.P., Plesnila, N., Pedroso de Lima, M.C., Wagner, E., Culmsee, C., 2008. Tf-lipoplexes for neuronal siRNA delivery: a promising system to mediate gene silencing in the CNS. *J Control Release* 132, 113–123. doi:10.1016/j.jconrel.2008.08.014
- Carroll, R.T., Bhatia, D., Geldenhuys, W., Bhatia, R., Miladore, N., Bishayee, A., Sutariya, V., 2010. Brain-targeted delivery of Tempol-loaded nanoparticles for neurological disorders. *J Drug Target* 18, 665–674. doi:10.3109/10611861003639796
- Carvey, P.M., Hendey, B., Monahan, A.J., 2009. The blood-brain barrier in neurodegenerative disease: a rhetorical perspective. *J. Neurochem.* 111, 291–314. doi:10.1111/j.1471-4159.2009.06319.x
- Carvey, P.M., Zhao, C.H., Hendey, B., Lum, H., Trachtenberg, J., Desai, B.S., Snyder, J., Zhu, Y.G., Ling, Z.D., 2005. 6-Hydroxydopamine-induced alterations in blood-brain barrier permeability. *Eur. J. Neurosci.* 22, 1158–1168. doi:10.1111/j.1460-9568.2005.04281.x
- Cerletti, A., Drewe, J., Fricker, G., Eberle, A.N., Huwyler, J., 2000. Endocytosis and transcytosis of an immunoliposome-based brain drug delivery system. *J Drug Target* 8, 435–446. doi:10.3109/10611860008997919
- Challis, R.C., Ravindra Kumar, S., Chan, K.Y., Challis, C., Beadle, K., Jang, M.J., Kim, H.M., Rajendran, P.S., Tompkins, J.D., Shivkumar, K., Deverman, B.E., Gradinaru, V., 2019. Systemic AAV vectors for widespread and targeted gene delivery in rodents. *Nat Protoc* 14, 379–414. doi:10.1038/s41596-018-0097-3
- Chandramohan, V., Sampson, J.H., Pastan, I., DD, B., 2017. Immunotoxin Therapy for Brain Tumors, in: *Experimental Brain Tumor Immunotherapies*. Elsevier . doi:10.1016/B978-0-12-802420-1.00010-7

- Chang, R., Knox, J., Chang, J., Derbedrossian, A., Vasilevko, V., Cribbs, D., Boado, R.J., Pardridge, W.M., Sumbria, R.K., 2017. Blood-Brain Barrier Penetrating Biologic TNF- $\alpha$  Inhibitor for Alzheimer's Disease. *Mol. Pharm.* 14, 2340–2349. doi:10.1021/acs.molpharmaceut.7b00200
- Chang, R., Maghribi, Al, A., Vanderpoel, V., Vasilevko, V., Cribbs, D.H., Boado, R., Pardridge, W.M., Sumbria, R.K., 2018. Brain Penetrating Bifunctional Erythropoietin-Transferrin Receptor Antibody Fusion Protein for Alzheimer's Disease. *Mol. Pharm.* 15, 4963–4973. doi:10.1021/acs.molpharmaceut.8b00594
- Charles, V., Mufson, E.J., Friden, P.M., Bartus, R.T., Kordower, J.H., 1996. Atrophy of cholinergic basal forebrain neurons following excitotoxic cortical lesions is reversed by intravenous administration of an NGF conjugate. *Brain Research* 728, 193–203.
- Chen, Z., Zhai, M., Xie, X., Zhang, Y., Ma, S., Li, Z., Yu, F., Zhao, B., Zhang, M., Yang, Y., Mei, X., 2017. Apoferritin Nanocage for Brain Targeted Doxorubicin Delivery. *Mol. Pharm.* 14, 3087–3097. doi:10.1021/acs.molpharmaceut.7b00341
- Cheng, M.J., Kumar, R., Sridhar, S., Webster, T.J., Ebong, E.E., 2016. Endothelial glycocalyx conditions influence nanoparticle uptake for passive targeting. *Int J Nanomedicine* 11, 3305–3315. doi:10.2147/IJN.S106299
- Cheng, Y., Zak, O., Aisen, P., Harrison, S.C., Walz, T., 2004. Structure of the human transferrin receptor-transferrin complex. *Cell* 116, 565–576.
- Cheng, Z., Zhang, J., Liu, H., Li, Y., Zhao, Y., Yang, E., 2010. Central nervous system penetration for small molecule therapeutic agents does not increase in multiple sclerosis- and Alzheimer's disease-related animal models despite reported blood-brain barrier disruption. *Drug Metab. Dispos.* 38, 1355–1361. doi:10.1124/dmd.110.033324
- Chevon, M., 1988. A site-specific mechanism for free radical induced biological damage: the essential role of redox-active transition metals. *Free Radic. Biol. Med.* 5, 27–37.
- Chiou, B., Connor, J.R., 2018. Emerging and Dynamic Biomedical Uses of Ferritin. *Pharmaceuticals (Basel)* 11, 124. doi:10.3390/ph11040124
- Chiou, B., Neal, E.H., Bowman, A.B., Lippmann, E.S., Simpson, I.A., Connor, J.R., 2018a. Endothelial cells are critical regulators of iron transport in a model of the human blood-brain barrier. *J. Cereb. Blood Flow Metab.* 22, 271678X18783372. doi:10.1177/0271678X18783372
- Chiou, B., Neal, E.H., Bowman, A.B., Lippmann, E.S., Simpson, I.A., Connor, J.R., 2018b. Pharmaceutical iron formulations do not cross a model of the human blood-brain barrier. *PLoS ONE* 13, e0198775. doi:10.1371/journal.pone.0198775
- Clark, A.J., Davis, M.E., 2015. Increased brain uptake of targeted nanoparticles by adding an acid-cleavable linkage between transferrin and the nanoparticle core. *Proc. Natl. Acad. Sci. U.S.A.* 201517048. doi:10.1073/pnas.1517048112

- Cohen, S., Milstein, C., 1967. Structure of antibody molecules. *Nature* 214, 449–52 passim.
- Connor, J.R., Ponnuru, P., Wang, X.-S., Patton, S.M., Allen, R.P., Earley, C.J., 2011. Profile of altered brain iron acquisition in restless legs syndrome. *Brain* 134, 959–968. doi:10.1093/brain/awr012
- Cornford, E.M., Hyman, S., Cornford, M.E., Chytrova, G., Rhee, J., Suzuki, T., Yamagata, T., Yamakawa, K., Penichet, M.L., Pardridge, W.M., 2015. Non-invasive gene targeting to the fetal brain after intravenous administration and transplacental transfer of plasmid DNA using PEGylated immunoliposomes. *J Drug Target* 1–10. doi:10.3109/1061186X.2015.1055569
- Couch, J.A., Yu, Y.J., Zhang, Y., Tarrant, J.M., Fuji, R.N., Meilandt, W.J., Solanoy, H., Tong, R.K., Hoyte, K., Luk, W., Lu, Y., Gadkar, K., Prabhu, S., Ordonia, B.A., Nguyen, Q., Lin, Y., Lin, Z., Balazs, M., Scarce-Levie, K., Ernst, J.A., Dennis, M.S., Watts, R.J., 2013. Addressing safety liabilities of TfR bispecific antibodies that cross the blood-brain barrier. *Sci Transl Med* 5, 183ra57–1–12. doi:10.1126/scitranslmed.3005338
- Crépin, R., Goenaga, A.-L., Jullienne, B., Bougherara, H., Legay, C., Benihoud, K., Marks, J.D., Poul, M.-A., 2010. Development of human single-chain antibodies to the transferrin receptor that effectively antagonize the growth of leukemias and lymphomas. *Cancer Research* 70, 5497–5506. doi:10.1158/0008-5472.CAN-10-0938
- Cui, L., Wang, Y., Liang, M., Chu, X., Fu, S., Gao, C., Liu, Q., Gong, W., Yang, M., Li, Z., Yu, L., Yang, C., Su, Z., Xie, X., Yang, Y., 2018. Dual-modified natural high density lipoprotein particles for systemic glioma-targeting drug delivery. *Drug Deliv* 25, 1865–1876. doi:10.1080/10717544.2018.1519002
- Cui, N., Lu, H., Li, M., 2018. Magnetic Nanoparticles Associated PEG/PLGA Block Copolymer Targeted with Anti-Transferrin Receptor Antibodies for Alzheimer's Disease. *J Biomed Nanotechnol* 14, 1017–1024. doi:10.1166/jbn.2018.2512
- Cummings, J.L., Cohen, S., van Dyck, C.H., Brody, M., Curtis, C., Cho, W., Ward, M., Friesenhahn, M., Rabe, C., Brunstein, F., Quartino, A., Honigberg, L.A., Fuji, R.N., Clayton, D., Mortensen, D., Ho, C., Paul, R., 2018. ABBY: A phase 2 randomized trial of crenezumab in mild to moderate Alzheimer disease. *Neurology* 90, e1889–e1897. doi:10.1212/WNL.0000000000005550
- Daneman, R., Zhou, L., Agalliu, D., Cahoy, J.D., Kaushal, A., Barres, B.A., 2010a. The mouse blood-brain barrier transcriptome: a new resource for understanding the development and function of brain endothelial cells. *PLoS ONE* 5, e13741. doi:10.1371/journal.pone.0013741
- Daneman, R., Zhou, L., Kebede, A.A., Barres, B.A., 2010b. Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* 468, 562–566. doi:10.1038/nature09513
- de Witte, W.E.A., Danhof, M., van der Graaf, P.H., de Lange, E.C.M., 2018. The implications of target saturation for the use of drug-target residence time. *Nat Rev Drug Discov* 18, 82–84. doi:10.1038/nrd.2018.234

- Deguchi, Y., Kurihara, A., Pardridge, W.M., 1999. Retention of biologic activity of human epidermal growth factor following conjugation to a blood-brain barrier drug delivery vector via an extended poly(ethylene glycol) linker. *Bioconjug. Chem.* 10, 32–37. doi:10.1021/bc9800522
- Denali Therapeutics, 2018. Denali Prospectus [WWW Document]. <https://www.sec.gov/Archives/edgar/data/adds.htm>. URL (accessed 1.7.19).
- Dennis, M.S., Watts, R.J., 2012. Transferrin antibodies into the brain. *Neuropsychopharmacology* 37, 302–303. doi:10.1038/npp.2011.196
- Desai Bradaric, B., Patel, A., Schneider, J.A., Carvey, P.M., Hendey, B., 2012. Evidence for angiogenesis in Parkinson's disease, incidental Lewy body disease, and progressive supranuclear palsy. *J Neural Transm* 119, 59–71. doi:10.1007/s00702-011-0684-8
- Desai, B.S., Monahan, A.J., Carvey, P.M., Hendey, B., 2007. Blood-brain barrier pathology in Alzheimer's and Parkinson's disease: implications for drug therapy. *Cell Transplant* 16, 285–299.
- Desai, B.S., Schneider, J.A., Li, J.-L., Carvey, P.M., Hendey, B., 2009. Evidence of angiogenic vessels in Alzheimer's disease. *J Neural Transm* 116, 587–597. doi:10.1007/s00702-009-0226-9
- Dickson, P.W., Aldred, A.R., Marley, P.D., Tu, G.F., Howlett, G.J., Schreiber, G., 1985. High prealbumin and transferrin mRNA levels in the choroid plexus of rat brain. *Biochem. Biophys. Res. Commun.* 127, 890–895.
- Ding, H., Sagar, V., Agudelo, M., Pilakka-Kanthikeel, S., Atluri, V.S.R., Raymond, A., Samikkannu, T., Nair, M.P., 2014. Enhanced blood-brain barrier transmigration using a novel transferrin embedded fluorescent magneto-liposome nanoformulation. *Nanotechnology* 25, 055101. doi:10.1088/0957-4484/25/5/055101
- Dixit, S., Miller, K., Zhu, Y., McKinnon, E., Novak, T., Kenney, M.E., Broome, A.-M., 2015a. Dual Receptor-Targeted Theranostic Nanoparticles for Localized Delivery and Activation of Photodynamic Therapy Drug in Glioblastomas. *Mol. Pharm.* 12, 3250–3260. doi:10.1021/acs.molpharmaceut.5b00216
- Dixit, S., Novak, T., Miller, K., Zhu, Y., Kenney, M.E., Broome, A.-M., 2015b. Transferrin receptor-targeted theranostic gold nanoparticles for photosensitizer delivery in brain tumors. *Nanoscale* 7, 1782–1790. doi:10.1039/c4nr04853a
- Do, T.M., Alata, W., Dodacki, A., Traversy, M.-T., Chacun, H., Pradier, L., Scherrmann, J.-M., Farinotti, R., Calon, F., Bourasset, F., 2014. Altered cerebral vascular volumes and solute transport at the blood-brain barriers of two transgenic mouse models of Alzheimer's disease. *Neuropharmacology* 81, 311–317. doi:10.1016/j.neuropharm.2014.02.010
- Doody, R.S., Thomas, R.G., Farlow, M., Iwatsubo, T., Vellas, B., Joffe, S., Kieburtz, K., Raman, R., Sun, X., Aisen, P.S., Siemers, E., Liu-Seifert, H., Mohs, R., Alzheimer's Disease Cooperative Study Steering Committee, Solanezumab Study Group, 2014. Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. *N Engl J Med* 370, 311–321. doi:10.1056/NEJMoa1312889

- Duck, K.A., Connor, J.R., 2016. Iron uptake and transport across physiological barriers. *Biometals* 29, 573–591. doi:10.1007/s10534-016-9952-2
- Duck, K.A., Simpson, I.A., Connor, J.R., 2017. Regulatory mechanisms for iron transport across the blood-brain barrier. *Biochem. Biophys. Res. Commun.* 494, 70–75. doi:10.1016/j.bbrc.2017.10.083
- Dziegielewska, K.M., Evans, C.A., New, H., Reynolds, M.L., Saunders, N.R., 1984. Synthesis of plasma proteins by rat fetal brain and choroid plexus. *Int. J. Dev. Neurosci.* 2, 215–222. doi:10.1016/0736-5748(84)90015-7
- Eckenroth, B.E., Steere, A.N., Chasteen, N.D., Everse, S.J., Mason, A.B., 2011. How the binding of human transferrin primes the transferrin receptor potentiating iron release at endosomal pH. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13089–13094. doi:10.1073/pnas.1105786108
- Eldridge, B.N., Bernish, B.W., Fahrenholtz, C.D., Singh, R., 2016. Photothermal therapy of glioblastoma multiforme using multiwalled carbon nanotubes optimized for diffusion in extracellular space. *ACS Biomater Sci Eng* 2, 963–976. doi:10.1021/acsbiomaterials.6b00052
- Enerson, B.E., Drewes, L.R., 2006. The rat blood-brain barrier transcriptome. *J. Cereb. Blood Flow Metab.* 26, 959–973. doi:10.1038/sj.jcbfm.9600249
- Falanga, A.P., Melone, P., Cagliani, R., Borbone, N., D'Errico, S., Piccialli, G., Netti, P.A., Guarnieri, D., 2018. Design, Synthesis and Characterization of Novel Co-Polymers Decorated with Peptides for the Selective Nanoparticle Transport across the Cerebral Endothelium. *Molecules* 23, 1655. doi:10.3390/molecules23071655
- Fan, K., Jia, X., Zhou, M., Wang, K., Conde, J., He, J., Tian, J., Yan, X., 2018. Ferritin Nanocarrier Traverses the Blood Brain Barrier and Kills Glioma. *ACS Nano* 12, acsnano.7b06969–4115. doi:10.1021/acsnano.7b06969
- Fang, X.T., Hultqvist, G., Meier, S.R., Antoni, G., Sehlin, D., Syvänen, S., 2019. High detection sensitivity with antibody-based PET radioligand for amyloid beta in brain. *Neuroimage* 184, 881–888. doi:10.1016/j.neuroimage.2018.10.011
- Faucheux, B.A., Hauw, J.J., Agid, Y., Hirsch, E.C., 1997. The density of [125I]-transferrin binding sites on perikarya of melanized neurons of the substantia nigra is decreased in Parkinson's disease. *Brain Research* 749, 170–174. doi:10.1016/s0006-8993(96)01412-6
- Faucheux, B.A., Hirsch, E.C., Villares, J., Selimi, F., Mouatt-Prigent, A., Javoy-Agid, F., Hauw, J.J., Agid, Y., 1993. Distribution of 125I-ferrotransferrin binding sites in the mesencephalon of control subjects and patients with Parkinson's disease. *J. Neurochem.* 60, 2338–2341. doi:10.1111/j.1471-4159.1993.tb03527.x
- Fishman, J.B., Rubin, J.B., Handrahan, J.V., Connor, J.R., Fine, R.E., 1987. Receptor-mediated transcytosis of transferrin across the blood-brain barrier. *J. Neurosci. Res.* 18, 299–304. doi:10.1002/jnr.490180206

- Foley, C.P., Nishimura, N., Neeves, K.B., Schaffer, C.B., Olbricht, W.L., 2012. Real-time imaging of perivascular transport of nanoparticles during convection-enhanced delivery in the rat cortex. *Ann Biomed Eng* 40, 292–303. doi:10.1007/s10439-011-0440-0
- Freskgård, P.-O., Urich, E., 2017. Antibody therapies in CNS diseases. *Neuropharmacology* 120, 38–55. doi:10.1016/j.neuropharm.2016.03.014
- Freskgård, P.O., Niewoehner, J., Urich, E., 2014. Time to open the blood-brain barrier gate for biologics? *Future Neurology*. doi:10.2217/fnl.14.15
- Friden, P.M., Olson, T.S., Obar, R., Walus, L.R., Putney, S.D., 1996. Characterization, receptor mapping and blood-brain barrier transcytosis of antibodies to the human transferrin receptor. *J. Pharmacol. Exp. Ther.* 278, 1491–1498.
- Friden, P.M., Walus, L., Taylor, M., Musso, G.F., Abelleira, S.A., Malfroy, B., Tehrani, F., Eckman, J.B., Morrow, A.R., Starzyk, R.M., 1992. Drug delivery to the brain using an anti-transferrin receptor antibody. *NIDA Res. Monogr.* 120, 202–217.
- Friden, P.M., Walus, L.R., Musso, G.F., Taylor, M.A., Malfroy, B., Starzyk, R.M., 1991. Anti-transferrin receptor antibody and antibody-drug conjugates cross the blood-brain barrier. *Proc. Natl. Acad. Sci. U.S.A.* 88, 4771–4775.
- Friden, P.M., Walus, L.R., Watson, P., Doctrow, S.R., Kozarich, J.W., Bäckman, C., Bergman, H., Hoffer, B., Bloom, F., Granholm, A.C., 1993. Blood-brain barrier penetration and in vivo activity of an NGF conjugate. *Science* 259, 373–377.
- Fu, A., Zhou, Q.-H., Hui, E.K.-W., Lu, J.Z., Boado, R.J., Pardridge, W.M., 2010. Intravenous treatment of experimental Parkinson's disease in the mouse with an IgG-GDNF fusion protein that penetrates the blood-brain barrier. *Brain Research* 1352, 208–213. doi:10.1016/j.brainres.2010.06.059
- Fujita, M., Lee, B.-S., Khazenzon, N.M., Penichet, M.L., Wawrowsky, K.A., Patil, R., Ding, H., Holler, E., Black, K.L., Ljubimova, J.Y., 2007. Brain tumor tandem targeting using a combination of monoclonal antibodies attached to biopoly(beta-L-malic acid). *J Control Release* 122, 356–363. doi:10.1016/j.jconrel.2007.05.032
- Fullstone, G., Nyberg, S., Tian, X., Battaglia, G., 2016. From the Blood to the Central Nervous System: A Nanoparticle's Journey Through the Blood-Brain Barrier by Transcytosis. *Int. Rev. Neurobiol.* 130, 41–72. doi:10.1016/bs.irn.2016.06.001
- Gatter, K.C., Brown, G., Trowbridge, I.S., Woolston, R.E., Mason, D.Y., 1983. Transferrin receptors in human tissues: their distribution and possible clinical relevance. *J. Clin. Pathol.* 36, 539–545.
- Giugliani, R., Giugliani, L., de Oliveira Poswar, F., Donis, K.C., Corte, A.D., Schmidt, M., Boado, R.J., Nestrasil, I., Nguyen, C., Chen, S., Pardridge, W.M., 2018. Neurocognitive and somatic stabilization in pediatric patients with severe Mucopolysaccharidosis Type I after 52 weeks of intravenous brain-

- penetrating insulin receptor antibody-iduronidase fusion protein (valanafusp alpha): an open label phase 1-2 trial. *Orphanet J Rare Dis* 13, 110. doi:10.1186/s13023-018-0849-8
- Gjetting, T., Jølck, R.I., Andresen, T.L., 2014. Effective nanoparticle-based gene delivery by a protease triggered charge switch. *Adv Healthc Mater* 3, 1107–1118. doi:10.1002/adhm.201300503
- Gomes, M.J., Kennedy, P.J., Martins, S., Sarmiento, B., 2017. Delivery of siRNA silencing P-gp in peptide-functionalized nanoparticles causes efflux modulation at the blood-brain barrier. *Nanomedicine (Lond)* 12, 1385–1399. doi:10.2217/nmm-2017-0023
- Gosk, S., Vermehren, C., Storm, G., Moos, T., 2004. Targeting anti-transferrin receptor antibody (OX26) and OX26-conjugated liposomes to brain capillary endothelial cells using in situ perfusion. *J. Cereb. Blood Flow Metab.* 24, 1193–1204. doi:10.1097/01.WCB.0000135592.28823.47
- Granhölm, A.C., Bäckman, C., Bloom, F., Ebendal, T., Gerhardt, G.A., Hoffer, B., Mackerlova, L., Olson, L., Söderström, S., Walus, L.R., 1994. NGF and anti-transferrin receptor antibody conjugate: short and long-term effects on survival of cholinergic neurons in intraocular septal transplants. *J. Pharmacol. Exp. Ther.* 268, 448–459.
- Gregori, M., Orlando, A., Re, F., Sesana, S., Nardo, L., Salerno, D., Mantegazza, F., Salvati, E., Zito, A., Malavasi, F., Masserini, M., Cazzaniga, E., 2016. Novel Antitransferrin Receptor Antibodies Improve the Blood-Brain Barrier Crossing Efficacy of Immunoliposomes. *J Pharm Sci* 105, 276–283. doi:10.1016/j.xphs.2015.11.009
- Gromnicova, R., Davies, H.A., Sreekanthreddy, P., Romero, I.A., Lund, T., Roitt, I.M., Phillips, J.B., Male, D.K., 2013. Glucose-coated gold nanoparticles transfer across human brain endothelium and enter astrocytes in vitro. *PLoS ONE* 8, e81043. doi:10.1371/journal.pone.0081043
- Gromnicova, R., Kaya, M., Romero, I.A., Williams, P., Satchell, S., Sharrack, B., Male, D., 2016a. Transport of Gold Nanoparticles by Vascular Endothelium from Different Human Tissues. *PLoS ONE* 11, e0161610. doi:10.1371/journal.pone.0161610
- Gromnicova, R., Yilmaz, C.U., Orhan, N., Kaya, M., Davies, H., Williams, P., Romero, I.A., Sharrack, B., Male, D., 2016b. Localization and mobility of glucose-coated gold nanoparticles within the brain. *Nanomedicine (Lond)* nmm.15.215. doi:10.2217/nmm.15.215
- Gu, J., Al-Bayati, K., Ho, E.A., 2017. Development of antibody-modified chitosan nanoparticles for the targeted delivery of siRNA across the blood-brain barrier as a strategy for inhibiting HIV replication in astrocytes. *Drug Deliv Transl Res* 7, 497–506. doi:10.1007/s13346-017-0368-5
- Gudbergsson, J.M., Kostrikov, S., Johnsen, K.B., Fliedner, F.P., Stolberg, C.B., Humle, N., Hansen, A.E., Kristensen, B.W., Christiansen, G., Kjaer, A., Andresen, T.L., Duroux, M., 2019. A tumorsphere model of glioblastoma multiforme with intratumoral heterogeneity for quantitative analysis of cellular migration and drug response. *Exp. Cell Res.* doi:10.1016/j.yexcr.2019.03.031

- Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L., Hediger, M.A., 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388, 482–488. doi:10.1038/41343
- Gustafsson, S., Sehlin, D., Lampa, E., Hammarlund-Udenaes, M., Loryan, I., 2019. Heterogeneous drug tissue binding in brain regions of rats, Alzheimer's patients and controls: impact on translational drug development. *Sci Rep* 9, 5308. doi:10.1038/s41598-019-41828-4
- Hadaczek, P., Yamashita, Y., Mirek, H., Tamas, L., Bohn, M.C., Noble, C., Park, J.W., Bankiewicz, K., 2006. The “perivascular pump” driven by arterial pulsation is a powerful mechanism for the distribution of therapeutic molecules within the brain. *Mol Ther* 14, 69–78. doi:10.1016/j.yymthe.2006.02.018
- Hagihara, N., Walbridge, S., Olson, A.W., Oldfield, E.H., Youle, R.J., 2000. Vascular protection by chloroquine during brain tumor therapy with Tf-CRM107. *Cancer Research* 60, 230–234.
- Hao, J., Bickel, U., 2013. Transferrin receptor mediated brain uptake during ischemia and reperfusion. *J Pharm Pharm Sci* 16, 541–550.
- Haqqani, A.S., Thom, G., Burrell, M., Delaney, C.E., Brunette, E., Baumann, E., Sodja, C., Jezierski, A., Webster, C., Stanimirovic, D.B., 2018. Intracellular sorting and transcytosis of the rat transferrin receptor antibody OX26 across the blood-brain barrier in vitro is dependent on its binding affinity. *J. Neurochem.* 146, 735–752. doi:10.1111/jnc.14482
- Haseloff, R.F., Dithmer, S., Winkler, L., Wolburg, H., Blasig, I.E., 2015. Transmembrane proteins of the tight junctions at the blood-brain barrier: structural and functional aspects. *Semin. Cell Dev. Biol.* 38, 16–25. doi:10.1016/j.semcdb.2014.11.004
- Hatakeyama, H., Akita, H., Maruyama, K., Suhara, T., Harashima, H., 2004. Factors governing the in vivo tissue uptake of transferrin-coupled polyethylene glycol liposomes in vivo. *Int J Pharm* 281, 25–33. doi:10.1016/j.ijpharm.2004.05.025
- Heggannavar, G.B., Hiremath, C.G., Achari, D.D., Pangarkar, V.G., Kariduraganavar, M.Y., 2018. Development of Doxorubicin-Loaded Magnetic Silica-Pluronic F-127 Nanocarriers Conjugated with Transferrin for Treating Glioblastoma across the Blood-Brain Barrier Using an in Vitro Model. *ACS Omega* 3, 8017–8026. doi:10.1021/acsomega.8b00152
- Hempel, C., Pasini, E.M., Kurtzhals, J.A.L., 2016. Endothelial Glycocalyx: Shedding Light on Malaria Pathogenesis. *Trends Mol Med* 22, 453–457. doi:10.1016/j.molmed.2016.04.004
- Hersom, M., Helms, H.C., Pretzer, N., Goldman, C., Jensen, A.I., Severin, G., Nielsen, M.S., Holm, R., Brodin, B., 2016. Transferrin receptor expression and role in transendothelial transport of transferrin in cultured brain endothelial monolayers. *Mol. Cell. Neurosci.* 76, 59–67. doi:10.1016/j.mcn.2016.08.009
- Heyes, J., Palmer, L., Bremner, K., MacLachlan, I., 2005. Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids. *J Control Release* 107, 276–287. doi:10.1016/j.jconrel.2005.06.014

- Hill, J.M., Ruff, M.R., Weber, R.J., Pert, C.B., 1985. Transferrin receptors in rat brain: neuropeptide-like pattern and relationship to iron distribution. *Proc. Natl. Acad. Sci. U.S.A.* 82, 4553–4557.
- Hirase, T., Staddon, J.M., Saitou, M., Ando-Akatsuka, Y., Itoh, M., Furuse, M., Fujimoto, K., Tsukita, S., Rubin, L.L., 1997. Occludin as a possible determinant of tight junction permeability in endothelial cells. *J. Cell. Sci.* 110 ( Pt 14), 1603–1613.
- Honig, L.S., Vellas, B., Woodward, M., Boada, M., Bullock, R., Borrie, M., Hager, K., Andreasen, N., Scarpini, E., Liu-Seifert, H., Case, M., Dean, R.A., Hake, A., Sundell, K., Poole Hoffmann, V., Carlson, C., Khanna, R., Mintun, M., Demattos, R., Selzler, K.J., Siemers, E., 2018. Trial of Solanezumab for Mild Dementia Due to Alzheimer's Disease. *N Engl J Med* 378, 321–330. doi:10.1056/NEJMoa1705971
- Hu, Y., Gaillard, P.J., Rip, J., de Lange, E.C.M., Hammarlund-Udenaes, M., 2018. In Vivo Quantitative Understanding of PEGylated Liposome's Influence on Brain Delivery of Diphenhydramine. *Mol. Pharm.* 15, 5493–5500. doi:10.1021/acs.molpharmaceut.8b00611
- Hu, Y., Rip, J., Gaillard, P.J., de Lange, E.C.M., Hammarlund-Udenaes, M., 2017. The Impact of Liposomal Formulations on the Release and Brain Delivery of Methotrexate: An In Vivo Microdialysis Study. *J Pharm Sci* 106, 2606–2613. doi:10.1016/j.xphs.2017.03.009
- Huebers, H.A., Finch, C.A., 1987. The physiology of transferrin and transferrin receptors. *Physiol. Rev.* 67, 520–582.
- Hultqvist, G., Syvänen, S., Fang, X.T., Lannfelt, L., Sehlin, D., 2017. Bivalent Brain Shuttle Increases Antibody Uptake by Monovalent Binding to the Transferrin Receptor. *Theranostics* 7, 308–318. doi:10.7150/thno.17155
- Hunt, D.W., Jiang, H., Levy, J.G., 1998. Photofrin increases murine spleen cell transferrin receptor expression and responsiveness to recombinant myeloid and erythroid growth factors. *Immunopharmacology* 38, 267–278.
- Huwyler, J., Cerletti, A., Fricker, G., Eberle, A.N., Drewe, J., 2002. By-passing of P-glycoprotein using immunoliposomes. *J Drug Target* 10, 73–79. doi:10.1080/10611860290007559
- Huwyler, J., Wu, D., Pardridge, W.M., 1996. Brain drug delivery of small molecules using immunoliposomes. *Proc. Natl. Acad. Sci. U.S.A.* 93, 14164–14169.
- Illiff, J.J., Wang, M., Liao, Y., Plogg, B.A., Peng, W., Gundersen, G.A., Benveniste, H., Vates, G.E., Deane, R., Goldman, S.A., Nagelhus, E.A., Nedergaard, M., 2012. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid  $\beta$ . *Sci Transl Med* 4, 147ra111–147ra111. doi:10.1126/scitranslmed.3003748
- Israel, L.L., Braubach, O., Galstyan, A., Chiechi, A., Shatalova, E.S., Grodzinski, Z., Ding, H., Black, K.L., Ljubimova, J.Y., Holler, E., 2019. A Combination of Tri-Leucine and Angiopep-2 Drives a Polyanionic Polymalic Acid Nanodrug Platform Across the Blood-Brain Barrier. *ACS Nano* 13, 1253–1271. doi:10.1021/acsnano.8b06437

- Jain, Atul, Jain, A., Garg, N.K., Tyagi, R.K., Singh, B., Katare, O.P., Webster, T.J., Soni, V., 2015. Surface engineered polymeric nanocarriers mediate the delivery of transferrin-methotrexate conjugates for an improved understanding of brain cancer. *Acta Biomater* 24, 140–151. doi:10.1016/j.actbio.2015.06.027
- Jain, Aviral, Chasoo, G., Singh, S.K., Saxena, A.K., Jain, S.K., 2011. Transferrin-appended PEGylated nanoparticles for temozolomide delivery to brain: in vitro characterisation. *J Microencapsul* 28, 21–28. doi:10.3109/02652048.2010.522257
- Jankovic, J., Goodman, I., Safirstein, B., Marmon, T.K., Schenk, D.B., Koller, M., Zago, W., Ness, D.K., Griffith, S.G., Grundman, M., Soto, J., Ostrowitzki, S., Boess, F.G., Martin-Facklam, M., Quinn, J.F., Isaacson, S.H., Omidvar, O., Ellenbogen, A., Kinney, G.G., 2018. Safety and Tolerability of Multiple Ascending Doses of PRX002/RG7935, an Anti- $\alpha$ -Synuclein Monoclonal Antibody, in Patients With Parkinson Disease: A Randomized Clinical Trial. *JAMA Neurol* 75, 1206–1214. doi:10.1001/jamaneurol.2018.1487
- Jefferies, W.A., Brandon, M.R., Hunt, S.V., Williams, A.F., Gatter, K.C., Mason, D.Y., 1984. Transferrin receptor on endothelium of brain capillaries. *Nature* 312, 162–163.
- Jefferies, W.A., Brandon, M.R., Williams, A.F., Hunt, S.V., 1985. Analysis of lymphopoietic stem cells with a monoclonal antibody to the rat transferrin receptor. *Immunology* 54, 333–341.
- Jeong Lee, H., Pardridge, W.M., 2000. Drug targeting to the brain using avidin-biotin technology in the mouse; (blood-brain barrier, monoclonal antibody, transferrin receptor, Alzheimer's disease). *J Drug Target* 8, 413–424. doi:10.3109/10611860008997917
- Jhaveri, A., Deshpande, P., Pattni, B., Torchilin, V., 2018. Transferrin-targeted, resveratrol-loaded liposomes for the treatment of glioblastoma. *J Control Release* 277, 89–101. doi:10.1016/j.jconrel.2018.03.006
- Jiang, C., Koyabu, N., Yonemitsu, Y., Shimazoe, T., Watanabe, S., Naito, M., Tsuruo, T., Ohtani, H., Sawada, Y., 2003. In vivo delivery of glial cell-derived neurotrophic factor across the blood-brain barrier by gene transfer into brain capillary endothelial cells. *Hum. Gene Ther.* 14, 1181–1191. doi:10.1089/104303403322168019
- Johnsen, K.B., 2018. On the use of the transferrin receptor as a target for brain drug delivery. Aalborg. doi:10.5278/vbn.phd.med.00108
- Johnsen, K.B., Bak, M., Kempen, P.J., Melander, F., Burkhart, A., Thomsen, M.S., Nielsen, M.S., Moos, T., Andresen, T.L., 2018. Antibody affinity and valency impact brain uptake of transferrin receptor-targeted gold nanoparticles. *Theranostics* 8, 3416–3436. doi:10.7150/thno.25228
- Johnsen, K.B., Bak, M., Melander, F., Thomsen, M.S., Burkhart, A., Kempen, P.J., Andresen, T.L., Moos, T., 2019. Modulating the antibody density changes the uptake and transport at the blood-brain barrier of both transferrin receptor-targeted gold nanoparticles and liposomal cargo. *J Control Release* 295, 237–249. doi:10.1016/j.jconrel.2019.01.005

- Johnsen, K.B., Burkhart, A., Melander, F., Kempen, P.J., Vejlebo, J.B., Siupka, P., Nielsen, M.S., Andresen, T.L., Moos, T., 2017. Targeting transferrin receptors at the blood-brain barrier improves the uptake of immunoliposomes and subsequent cargo transport into the brain parenchyma. *Sci Rep* 7, 437. doi:10.1038/s41598-017-11220-1
- Johnsen, K.B., Moos, T., 2016. Revisiting nanoparticle technology for blood-brain barrier transport: Unfolding at the endothelial gate improves the fate of transferrin receptor-targeted liposomes. *J Control Release* 222, 32–46. doi:10.1016/j.jconrel.2015.11.032
- Kaasgaard, T., Andresen, T.L., 2010. Liposomal cancer therapy: exploiting tumor characteristics. *Expert Opin Drug Deliv* 7, 225–243. doi:10.1517/17425240903427940
- Kalaria, R.N., Sromek, S.M., Grahovac, I., Harik, S.I., 1992. Transferrin receptors of rat and human brain and cerebral microvessels and their status in Alzheimer's disease. *Brain Research* 585, 87–93. doi:10.1016/0006-8993(92)91193-i
- Kang, T., Jiang, M., Jiang, D., Feng, X., Yao, J., Song, Q., Chen, H., Gao, X., Chen, J., 2015. Enhancing Glioblastoma-Specific Penetration by Functionalization of Nanoparticles with an Iron-Mimic Peptide Targeting Transferrin/Transferrin Receptor Complex. *Mol. Pharm.* 12, 2947–2961. doi:10.1021/acs.molpharmaceut.5b00222
- Kang, Y.-S., Jung, H.-J., Oh, J.-S., Song, D.-Y., 2016. Use of PEGylated Immunoliposomes to Deliver Dopamine Across the Blood-Brain Barrier in a Rat Model of Parkinson's Disease. *CNS Neurosci Ther* 22, 817–823. doi:10.1111/cns.12580
- Kang, Y.S., Bickel, U., Pardridge, W.M., 1994. Pharmacokinetics and saturable blood-brain barrier transport of biotin bound to a conjugate of avidin and a monoclonal antibody to the transferrin receptor. *Drug Metab. Dispos.* 22, 99–105.
- Kang, Y.S., Boado, R.J., Pardridge, W.M., 1995. Pharmacokinetics and organ clearance of a 3'-biotinylated, internally [32P]-labeled phosphodiester oligodeoxynucleotide coupled to a neutral avidin/monoclonal antibody conjugate. *Drug Metab. Dispos.* 23, 55–59.
- Kang, Y.S., Pardridge, W.M., 1994. Use of neutral avidin improves pharmacokinetics and brain delivery of biotin bound to an avidin-monoclonal antibody conjugate. *J. Pharmacol. Exp. Ther.* 269, 344–350.
- Kang, Y.S., Voigt, K., Bickel, U., 2000. Stability of the disulfide bond in an avidin-biotin linked chimeric peptide during in vivo transcytosis through brain endothelial cells. *J Drug Target* 8, 425–434. doi:10.3109/10611860008997918
- Karaoglu Hanzatian, D., Schwartz, A., Gizatullin, F., Erickson, J., Deng, K., Villanueva, R., Stedman, C., Harris, C., Ghayur, T., Goodearl, A., 2018. Brain uptake of multivalent and multi-specific DVD-Ig proteins after systemic administration. *MAbs* 10, 765–777. doi:10.1080/19420862.2018.1465159
- Karatas, H., Aktaş, Y., Gürsoy-Özdemir, Y., Bodur, E., Yemisci, M., Caban, S., Vural, A., Pinarbasli, O., Capan, Y., Fernandez-Megia, E., Novoa-Carballal, R., Riguera, R., Andrieux, K., Couvreur, P., Dalkara,

- T., 2009. A nanomedicine transports a peptide caspase-3 inhibitor across the blood-brain barrier and provides neuroprotection. *Journal of Neuroscience* 29, 13761–13769. doi:10.1523/JNEUROSCI.4246-09.2009
- Kawabata, H., Germain, R.S., Vuong, P.T., Nakamaki, T., Said, J.W., Koeffler, H.P., 2000. Transferrin receptor 2-alpha supports cell growth both in iron-chelated cultured cells and in vivo. *J. Biol. Chem.* 275, 16618–16625. doi:10.1074/jbc.M908846199
- Kedmi, R., Veiga, N., Ramishetti, S., Goldsmith, M., Rosenblum, D., Dammes, N., Hazan-Halevy, I., Nahary, L., Leviatan-Ben-Arye, S., Harlev, M., Behlke, M., Benhar, I., Lieberman, J., Peer, D., 2018. A modular platform for targeted RNAi therapeutics. *Nat Nanotechnol* 13, 214–219. doi:10.1038/s41565-017-0043-5
- Kempen, P.J., Thakor, A.S., Zavaleta, C., Gambhir, S.S., Sinclair, R., 2013. A scanning transmission electron microscopy approach to analyzing large volumes of tissue to detect nanoparticles. *Microsc. Microanal.* 19, 1290–1297. doi:10.1017/S143192761300192X
- Khan, A.I., Liu, J., Dutta, P., 2018. Iron transport kinetics through blood-brain barrier endothelial cells. *Biochim Biophys Acta Gen Subj* 1862, 1168–1179. doi:10.1016/j.bbagen.2018.02.010
- Kim, J.-S., Choi, D.-K., Shin, J.-Y., Shin, S.-M., Park, S.-W., Cho, H.-S., Kim, Y.-S., 2016. Endosomal acidic pH-induced conformational changes of a cytosol-penetrating antibody mediate endosomal escape. *J Control Release.* doi:10.1016/j.jconrel.2016.05.066
- Kizelsztejn, P., Ovadia, H., Garbuzenko, O., Sigal, A., Barenholz, Y., 2009. Pegylated nanoliposomes remote-loaded with the antioxidant tempamine ameliorate experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 213, 20–25. doi:10.1016/j.jneuroim.2009.05.019
- Klomp, L.W., Farhangrazi, Z.S., Dugan, L.L., Gitlin, J.D., 1996. Ceruloplasmin gene expression in the murine central nervous system. *J. Clin. Invest.* 98, 207–215. doi:10.1172/JCI118768
- Kordower, J.H., Charles, V., Bayer, R., Bartus, R.T., Putney, S., Walus, L.R., Friden, P.M., 1994. Intravenous administration of a transferrin receptor antibody-nerve growth factor conjugate prevents the degeneration of cholinergic striatal neurons in a model of Huntington disease. *Proc. Natl. Acad. Sci. U.S.A.* 91, 9077–9080.
- Krauze, M.T., Forsayeth, J., Park, J.W., Bankiewicz, K.S., 2006. Real-time imaging and quantification of brain delivery of liposomes. *Pharm. Res.* 23, 2493–2504. doi:10.1007/s11095-006-9103-5
- Krauze, M.T., Saito, R., Noble, C., Bringas, J., Forsayeth, J., McKnight, T.R., Park, J., Bankiewicz, K.S., 2005. Effects of the perivascular space on convection-enhanced delivery of liposomes in primate putamen. *Exp. Neurol.* 196, 104–111. doi:10.1016/j.expneurol.2005.07.009
- Kreuter, J., 2014. Drug delivery to the central nervous system by polymeric nanoparticles: what do we know? *Adv. Drug Deliv. Rev.* 71, 2–14. doi:10.1016/j.addr.2013.08.008

- Kuang, Y., An, S., Guo, Y., Huang, S., Shao, K., Liu, Y., Li, J., Ma, H., Jiang, C., 2013. T7 peptide-functionalized nanoparticles utilizing RNA interference for glioma dual targeting. *Int J Pharm* 454, 11–20. doi:10.1016/j.ijpharm.2013.07.019
- Kuang, Y., Jiang, X., Zhang, Y., Lu, Y., Ma, H., Guo, Y., Zhang, Y., An, S., Li, J., Liu, L., Wu, Y., Liang, J., Jiang, C., 2016. Dual Functional Peptide-Driven Nanoparticles for Highly Efficient Glioma-Targeting and Drug Codelivery. *Mol. Pharm.* 13, 1599–1607. doi:10.1021/acs.molpharmaceut.6b00051
- Kuo, Y.-C., Lin, P.-I., Wang, C.-C., 2011. Targeting nevirapine delivery across human brain microvascular endothelial cells using transferrin-grafted poly(lactide-co-glycolide) nanoparticles. *Nanomedicine (Lond)* 6, 1011–1026. doi:10.2217/nmm.11.25
- Kutuzov, N., Flyvbjerg, H., Lauritzen, M., 2018. Contributions of the glycocalyx, endothelium, and extravascular compartment to the blood-brain barrier. *Proc. Natl. Acad. Sci. U.S.A.* 115, E9429–E9438. doi:10.1073/pnas.1802155115
- Kverneland, A.H., Enevold, C., Donia, M., Bastholt, L., Svane, I.M., Nielsen, C.H., 2018. Development of anti-drug antibodies is associated with shortened survival in patients with metastatic melanoma treated with ipilimumab. *Oncoimmunology* 7, e1424674. doi:10.1080/2162402X.2018.1424674
- Labrijn, A.F., Janmaat, M.L., Reichert, J.M., Parren, P.W.H.I., 2019. Bispecific antibodies: a mechanistic review of the pipeline. *Nat Rev Drug Discov* 132, 1770. doi:10.1038/s41573-019-0028-1
- Ladokhin, A.S., Wimley, W.C., White, S.H., 1995. Leakage of membrane vesicle contents: determination of mechanism using fluorescence reuquenching. *Biophys. J.* 69, 1964–1971. doi:10.1016/S0006-3495(95)80066-4
- Lakkadwala, S., Singh, J., 2019. Co-delivery of doxorubicin and erlotinib through liposomal nanoparticles for glioblastoma tumor regression using an in vitro brain tumor model. *Colloids Surf B Biointerfaces* 173, 27–35. doi:10.1016/j.colsurfb.2018.09.047
- Larrick, J.W., Hyman, E.S., 1984. Acquired iron-deficiency anemia caused by an antibody against the transferrin receptor. *N Engl J Med* 311, 214–218. doi:10.1056/NEJM198407263110402
- Laske, D.W., Youle, R.J., Oldfield, E.H., 1997. Tumor regression with regional distribution of the targeted toxin TF-CRM107 in patients with malignant brain tumors. *Nat. Med.* 3, 1362–1368.
- Lawrence, C.M., Ray, S., Babyonyshev, M., Galluser, R., Borhani, D.W., Harrison, S.C., 1999. Crystal structure of the ectodomain of human transferrin receptor. *Science* 286, 779–782.
- Lee, H.J., Boado, R.J., Braasch, D.A., Corey, D.R., Pardridge, W.M., 2002. Imaging gene expression in the brain in vivo in a transgenic mouse model of Huntington's disease with an antisense radiopharmaceutical and drug-targeting technology. *J. Nucl. Med.* 43, 948–956.
- Lee, H.J., Engelhardt, B., Lesley, J., Bickel, U., Pardridge, W.M., 2000. Targeting rat anti-mouse transferrin receptor monoclonal antibodies through blood-brain barrier in mouse. *J. Pharmacol. Exp. Ther.* 292, 1048–1052.

- Leitner, D.F., Connor, J.R., 2012. Functional roles of transferrin in the brain. *Biochim. Biophys. Acta* 1820, 393–402. doi:10.1016/j.bbagen.2011.10.016
- Lesley, J., Schulte, R., Woods, J., 1989. Modulation of transferrin receptor expression and function by anti-transferrin receptor antibodies and antibody fragments. *Exp. Cell Res.* 182, 215–233.
- Li, J.Y., Sugimura, K., Boado, R.J., Lee, H.J., Zhang, C., Duebel, S., Pardridge, W.M., 1999. Genetically engineered brain drug delivery vectors: cloning, expression and in vivo application of an anti-transferrin receptor single chain antibody-streptavidin fusion gene and protein. *12*, 787–796.
- Li, L., Di, X., Zhang, S., Kan, Q., Liu, H., Lu, T., Wang, Y., Fu, Q., Sun, J., He, Z., 2016. Large amino acid transporter 1 mediated glutamate modified docetaxel-loaded liposomes for glioma targeting. *Colloids Surf B Biointerfaces* 141, 260–267. doi:10.1016/j.colsurfb.2016.01.041
- Li, S., Peng, Z., Dallman, J., Baker, J., Othman, A.M., Blackwelder, P.L., Leblanc, R.M., 2016. Crossing the blood-brain-barrier with transferrin conjugated carbon dots: A zebrafish model study. *Colloids Surf B Biointerfaces* 145, 251–256. doi:10.1016/j.colsurfb.2016.05.007
- Li, X.B., Liao, G.S., Shu, Y.Y., Tang, S.X., 2000. Brain delivery of biotinylated NGF bounded to an avidin-transferrin conjugate. *J Nat Toxins* 9, 73–83.
- Liang, M., Gao, C., Wang, Y., Gong, W., Fu, S., Cui, L., Zhou, Z., Chu, X., Zhang, Y., Liu, Q., Zhao, X., Zhao, B., Yang, M., Li, Z., Yang, C., Xie, X., Yang, Y., Gao, C., 2018. Enhanced blood-brain barrier penetration and glioma therapy mediated by T7 peptide-modified low-density lipoprotein particles. *Drug Deliv* 25, 1652–1663. doi:10.1080/10717544.2018.1494223
- Liao, F., Li, A., Xiong, M., Bien-Ly, N., Jiang, H., Zhang, Y., Finn, M.B., Hoyle, R., Keyser, J., Lefton, K.B., Robinson, G.O., Serrano, J.R., Silverman, A.P., Guo, J.L., Getz, J., Henne, K., Leyns, C.E., Gallardo, G., Ulrich, J.D., Sullivan, P.M., Lerner, E.P., Hudry, E., Sweeney, Z.K., Dennis, M.S., Hyman, B.T., Watts, R.J., Holtzman, D.M., 2018. Targeting of nonlipidated, aggregated apoE with antibodies inhibits amyloid accumulation. *J. Clin. Invest.* 128, 2144–2155. doi:10.1172/JCI96429
- Lichota, J., Skjørringe, T., Thomsen, L.B., Moos, T., 2010. Macromolecular drug transport into the brain using targeted therapy. *J. Neurochem.* 113, 1–13. doi:10.1111/j.1471-4159.2009.06544.x
- Lilius, T.O., Blomqvist, K., Hauglund, N.L., Liu, G., Stæger, F.F., Bærentzen, S., Du, T., Ahlström, F., Backman, J.T., Kalso, E.A., Rauhala, P.V., Nedergaard, M., 2019. Dexmedetomidine enhances glymphatic brain delivery of intrathecally administered drugs. *J Control Release* 304, 29–38. doi:10.1016/j.jconrel.2019.05.005
- Lilly Announces Top-Line Results of Solanezumab Phase 3 Clinical Trial | Eli Lilly and Company, 2016. Lilly Announces Top-Line Results of Solanezumab Phase 3 Clinical Trial | Eli Lilly and Company.
- Lindqvist, A., Fridén, M., Hammarlund-Udenaes, M., 2016. Pharmacokinetic considerations of nanodelivery to the brain: Using modeling and simulations to predict the outcome of liposomal formulations. *Eur J Pharm Sci* 92, 173–182. doi:10.1016/j.ejps.2016.07.003

- Liu, C., Liu, X.-N., Wang, G.-L., Hei, Y., Meng, S., Yang, L.-F., Yuan, L., Xie, Y., 2017. A dual-mediated liposomal drug delivery system targeting the brain: rational construction, integrity evaluation across the blood-brain barrier, and the transporting mechanism to glioma cells. *Int J Nanomedicine* 12, 2407–2425. doi:10.2147/IJN.S131367
- Liu, G., Shen, H., Mao, J., Zhang, L., Jiang, Z., Sun, T., Lan, Q., Zhang, Z., 2013. Transferrin modified graphene oxide for glioma-targeted drug delivery: in vitro and in vivo evaluations. *ACS Appl Mater Interfaces* 5, 6909–6914. doi:10.1021/am402128s
- Liu, J., Weller, G.E.R., Zern, B., Ayyaswamy, P.S., Eckmann, D.M., Muzykantov, V.R., Radhakrishnan, R., 2010. Computational model for nanocarrier binding to endothelium validated using in vivo, in vitro, and atomic force microscopy experiments. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16530–16535. doi:10.1073/pnas.1006611107
- Liu-Seifert, H., Siemers, E., Holdridge, K.C., Andersen, S.W., Lipkovich, I., Carlson, C., Sethuraman, G., Hoog, S., Hayduk, R., Doody, R., Aisen, P., 2015. Delayed-start analysis: Mild Alzheimer's disease patients in solanezumab trials, 3.5 years. *Alzheimer's & Dementia: Translational Research & Clinical Interventions* 1, 111–121. doi:10.1016/j.trci.2015.06.006
- Lo, M., Kim, H.S., Tong, R.K., Bainbridge, T.W., Vernes, J.-M., Zhang, Y., Lin, Y.L., Chung, S., Dennis, M.S., Zuchero, Y.J.Y., Watts, R.J., Couch, J.A., Meng, Y.G., Atwal, J.K., Brezski, R.J., Spiess, C., Ernst, J.A., 2017. Effector-attenuating Substitutions That Maintain Antibody Stability and Reduce Toxicity in Mice. *J. Biol. Chem.* 292, 3900–3908. doi:10.1074/jbc.M116.767749
- Logovinsky, V., Satlin, A., Lai, R., Swanson, C., Kaplow, J., Osswald, G., Basun, H., Lannfelt, L., 2016. Safety and tolerability of BAN2401--a clinical study in Alzheimer's disease with a protofibril selective A $\beta$  antibody. *Alzheimers Res Ther* 8, 14. doi:10.1186/s13195-016-0181-2
- Loureiro, J.A., Gomes, B., Fricker, G., Coelho, M.A.N., Rocha, S., Pereira, M.C., 2016. Cellular uptake of PLGA nanoparticles targeted with anti-amyloid and anti-transferrin receptor antibodies for Alzheimer's disease treatment. *Colloids Surf B Biointerfaces* 145, 8–13. doi:10.1016/j.colsurfb.2016.04.041
- Lu, J.P., Hayashi, K., Awai, M., 1989. Transferrin receptor expression in normal, iron-deficient and iron-overloaded rats. *Acta Pathol. Jpn.* 39, 759–764.
- Lu, L.-N., Qian, Z.M., Wu, K.-C., Yung, W.-H., Ke, Y., 2017. Expression of Iron Transporters and Pathological Hallmarks of Parkinson's and Alzheimer's Diseases in the Brain of Young, Adult, and Aged Rats. *Mol Neurobiol* 54, 5213–5224. doi:10.1007/s12035-016-0067-0
- Luck, A.N., Mason, A.B., 2013. Structure and dynamics of drug carriers and their interaction with cellular receptors: focus on serum transferrin. *Adv. Drug Deliv. Rev.* 65, 1012–1019. doi:10.1016/j.addr.2012.11.001
- Luck, A.N., Mason, A.B., 2012. Transferrin-mediated cellular iron delivery. *Curr Top Membr* 69, 3–35. doi:10.1016/B978-0-12-394390-3.00001-X

- Ma, Q., Ries, M., Decker, Y., Müller, A., Riner, C., Bücker, A., Fassbender, K., Detmar, M., Proulx, S.T., 2019. Rapid lymphatic efflux limits cerebrospinal fluid flow to the brain. *Acta Neuropathol.* 137, 151–165. doi:10.1007/s00401-018-1916-x
- Macdonald, J., Henri, J., Goodman, L., Xiang, D., Duan, W., Shigdar, S., 2017. Development of a Bifunctional Aptamer Targeting the Transferrin Receptor and Epithelial Cell Adhesion Molecule (EpCAM) for the Treatment of Brain Cancer Metastases. *ACS Chem Neurosci* 8, 777–784. doi:10.1021/acscchemneuro.6b00369
- Macdonald, J., Houghton, P., Xiang, D., Duan, W., Shigdar, S., 2016. Truncation and Mutation of a Transferrin Receptor Aptamer Enhances Binding Affinity. *Nucleic Acid Ther* 26, 348–354. doi:10.1089/nat.2015.0585
- MacKay, J.A., Deen, D.F., Szoka, F.C., 2005. Distribution in brain of liposomes after convection enhanced delivery; modulation by particle charge, particle diameter, and presence of steric coating. *Brain Research* 1035, 139–153. doi:10.1016/j.brainres.2004.12.007
- Mahajan, S.D., Roy, I., Xu, G., Yong, K.-T., Ding, H., Aalinkeel, R., Reynolds, J., Sykes, D., Nair, B.B., Lin, E.Y., Prasad, P.N., Schwartz, S.A., 2010. Enhancing the delivery of anti retroviral drug “Saquinavir” across the blood brain barrier using nanoparticles. *Curr. HIV Res.* 8, 396–404.
- Male, D., Gromnicova, R., McQuaid, C., 2016. Gold Nanoparticles for Imaging and Drug Transport to the CNS. *Int. Rev. Neurobiol.* 130, 155–198. doi:10.1016/bs.irn.2016.05.003
- Malik, I.A., Naz, N., Sheikh, N., Khan, S., Moriconi, F., Blaschke, M., Ramadori, G., 2011. Comparison of changes in gene expression of transferrin receptor-1 and other iron-regulatory proteins in rat liver and brain during acute-phase response. *Cell Tissue Res.* 344, 299–312. doi:10.1007/s00441-011-1152-3
- Manich, G., Cabezón, I., del Valle, J., Duran-Vilaregut, J., Camins, A., Pallàs, M., Pelegrí, C., Vilaplana, J., 2013. Study of the transcytosis of an anti-transferrin receptor antibody with a Fab' cargo across the blood-brain barrier in mice. *Eur J Pharm Sci* 49, 556–564. doi:10.1016/j.ejps.2013.05.027
- Mariano, C., Palmela, I., Pereira, P., Fernandes, A., Falcão, A.S., Cardoso, F.L., Vaz, A.R., Campos, A.R., Gonçalves-Ferreira, A., Kim, K.S., Brites, D., Brito, M.A., 2013. Tricellulin expression in brain endothelial and neural cells. *Cell Tissue Res.* 351, 397–407. doi:10.1007/s00441-012-1529-y
- Markoutsas, E., Papadia, K., Giannou, A.D., Spella, M., Cagnotto, A., Salmona, M., Stathopoulos, G.T., Antimisiaris, S.G., 2014. Mono and dually decorated nanoliposomes for brain targeting, in vitro and in vivo studies. *Pharm. Res.* 31, 1275–1289. doi:10.1007/s11095-013-1249-3
- Martell, L.A., Agrawal, A., Ross, D.A., Muraszko, K.M., 1993. Efficacy of transferrin receptor-targeted immunotoxins in brain tumor cell lines and pediatric brain tumors. *Cancer Research* 53, 1348–1353.
- Mash, D.C., Pablo, J., Flynn, D.D., Efang, S.M., Weiner, W.J., 1990. Characterization and distribution of transferrin receptors in the rat brain. *J. Neurochem.* 55, 1972–1979.

- Mason, A.B., Byrne, S.L., Everse, S.J., Roberts, S.E., Chasteen, N.D., Smith, V.C., MacGillivray, R.T.A., Kandemir, B., Bou-Abdallah, F., 2009. A loop in the N-lobe of human serum transferrin is critical for binding to the transferrin receptor as revealed by mutagenesis, isothermal titration calorimetry, and epitope mapping. *J. Mol. Recognit.* 22, 521–529. doi:10.1002/jmr.979
- Mayle, K.M., Le, A.M., Kamei, D.T., 2012. The intracellular trafficking pathway of transferrin. *Biochim. Biophys. Acta* 1820, 264–281. doi:10.1016/j.bbagen.2011.09.009
- McCarthy, R.C., Kosman, D.J., 2013. Ferroportin and exocytosomal ferroxidase activity are required for brain microvascular endothelial cell iron efflux. *J. Biol. Chem.* 288, 17932–17940. doi:10.1074/jbc.M113.455428
- McCarthy, R.C., Kosman, D.J., 2012. Mechanistic analysis of iron accumulation by endothelial cells of the BBB. *Biomaterials* 25, 665–675. doi:10.1007/s10534-012-9538-6
- McConnell, E.M., Ventura, K., Dwyer, Z., Hunt, V., Koudrina, A., Holahan, M.R., DeRosa, M.C., 2018. In Vivo Use of a Multi-DNA Aptamer-Based Payload/Targeting System To Study Dopamine Dysregulation in the Central Nervous System. *ACS Chem Neurosci* 10, 371–383. doi:10.1021/acschemneuro.8b00292
- Mead, B.P., Mastorakos, P., Suk, J.S., Klivanov, A.L., Hanes, J., Price, R.J., 2016. Targeted gene transfer to the brain via the delivery of brain-penetrating DNA nanoparticles with focused ultrasound. *J Control Release* 223, 109–117. doi:10.1016/j.jconrel.2015.12.034
- Meier, S.R., Syvänen, S., Hultqvist, G., Fang, X.T., Roshanbin, S., Lannfelt, L., Neumann, U., Sehlin, D., 2018. Antibody-Based In Vivo PET Imaging Detects Amyloid- $\beta$  Reduction in Alzheimer Transgenic Mice After BACE-1 Inhibition. *J. Nucl. Med.* 59, 1885–1891. doi:10.2967/jnumed.118.213140
- Minh Do, T., Dodacki, A., Alata, W., Calon, F., Nicolic, S., Scherrmann, J.-M., Farinotti, R., Bourasset, F., 2015. Age-Dependent Regulation of the Blood-Brain Barrier Influx/Efflux Equilibrium of Amyloid- $\beta$  Peptide in a Mouse Model of Alzheimer's Disease (3xTg-AD). *J Alzheimers Dis.* doi:10.3233/JAD-150350
- Montagne, A., Barnes, S.R., Sweeney, M.D., Halliday, M.R., Sagare, A.P., Zhao, Z., Toga, A.W., Jacobs, R.E., Liu, C.Y., Amezcua, L., Harrington, M.G., Chui, H.C., Law, M., Zlokovic, B.V., 2015. Blood-brain barrier breakdown in the aging human hippocampus. *Neuron* 85, 296–302. doi:10.1016/j.neuron.2014.12.032
- Montemiglio, L.C., Testi, C., Ceci, P., Falvo, E., Pitea, M., Savino, C., Arcovito, A., Peruzzi, G., Baiocco, P., Mancina, F., Boffi, A., Georges, des, A., Vallone, B., 2019. Cryo-EM structure of the human ferritin-transferrin receptor 1 complex. *Nat Commun* 10, 1121. doi:10.1038/s41467-019-09098-w
- Moos, T., 1996. Immunohistochemical localization of intraneuronal transferrin receptor immunoreactivity in the adult mouse central nervous system. *J. Comp. Neurol.* 375, 675–692. doi:10.1002/(SICI)1096-9861(19961125)375:4<675::AID-CNE8>3.0.CO;2-Z

- Moos, T., 1995. Developmental profile of non-heme iron distribution in the rat brain during ontogenesis. *Brain Res. Dev. Brain Res.* 87, 203–213. doi:10.1016/0165-3806(95)00077-Q
- Moos, T., Morgan, E.H., 2004. The significance of the mutated divalent metal transporter (DMT1) on iron transport into the Belgrade rat brain. *J. Neurochem.* 88, 233–245.
- Moos, T., Morgan, E.H., 2001. Restricted transport of anti-transferrin receptor antibody (OX26) through the blood-brain barrier in the rat. *J. Neurochem.* 79, 119–129.
- Moos, T., Morgan, E.H., 2000. Transferrin and transferrin receptor function in brain barrier systems. *Cell. Mol. Biol. (Noisy-le-grand)* 20, 77–95. doi:10.1023/A:1006948027674
- Moos, T., Oates, P.S., Morgan, E.H., 1998. Expression of the neuronal transferrin receptor is age dependent and susceptible to iron deficiency. *J. Comp. Neurol.* 398, 420–430. doi:10.1002/(SICI)1096-9861(19980831)398:3<420::AID-CNE8>3.0.CO;2-1
- Moos, T., Rosengren Nielsen, T., Skjørringe, T., Morgan, E.H., 2007. Iron trafficking inside the brain. *J. Neurochem.* 103, 1730–1740. doi:10.1111/j.1471-4159.2007.04976.x
- Moos, T., Skjørringe, T., Gosk, S., Morgan, E.H., 2006. Brain capillary endothelial cells mediate iron transport into the brain by segregating iron from transferrin without the involvement of divalent metal transporter 1. *J. Neurochem.* 98, 1946–1958. doi:10.1111/j.1471-4159.2006.04023.x
- Moos, T., Trinder, D., Morgan, E.H., 2000. Cellular distribution of ferric iron, ferritin, transferrin and divalent metal transporter 1 (DMT1) in substantia nigra and basal ganglia of normal and beta2-microglobulin deficient mouse brain. *Cell. Mol. Biol. (Noisy-le-grand)* 46, 549–561.
- Morgan, E.H., 1983. Effect of pH and iron content of transferrin on its binding to reticulocyte receptors. *Biochim. Biophys. Acta* 762, 498–502.
- Morgan, E.H., 1979. Studies on the mechanism of iron release from transferrin. *Biochim. Biophys. Acta* 580, 312–326.
- Morris, A.W.J., Sharp, M.M., Albargothy, N.J., Fernandes, R., Hawkes, C.A., Verma, A., Weller, R.O., Carare, R.O., 2016. Vascular basement membranes as pathways for the passage of fluid into and out of the brain. *Acta Neuropathol.* 131, 725–736. doi:10.1007/s00401-016-1555-z
- Morris, C.M., Candy, J.M., Kerwin, J.M., Edwardson, J.A., 1994a. Transferrin receptors in the normal human hippocampus and in Alzheimer's disease. *Neuropathol. Appl. Neurobiol.* 20, 473–477.
- Morris, C.M., Candy, J.M., Oakley, A.E., Taylor, G.A., Mountfort, S., Bishop, H., Ward, M.K., Bloxham, C.A., Edwardson, J.A., 1989. Comparison of the regional distribution of transferrin receptors and aluminium in the forebrain of chronic renal dialysis patients. *J. Neurol. Sci.* 94, 295–306.
- Morris, C.M., Candy, J.M., Omar, S., Bloxham, C.A., Edwardson, J.A., 1994b. Transferrin receptors in the parkinsonian midbrain. *Neuropathol. Appl. Neurobiol.* 20, 468–472.
- Morris, C.M., Keith, A.B., Edwardson, J.A., Pullen, R.G., 1992. Uptake and distribution of iron and transferrin in the adult rat brain. *J. Neurochem.* 59, 300–306.

- Möckl, L., Hirn, S., Torrano, A.A., Uhl, B., Bräuchle, C., Krombach, F., 2017. The glycocalyx regulates the uptake of nanoparticles by human endothelial cells in vitro. *Nanomedicine (Lond)*. doi:10.2217/nmm-2016-0332
- Mu, C., Dave, N., Hu, J., Desai, P., Pauletti, G., Bai, S., Hao, J., 2013. Solubilization of flurbiprofen into aptamer-modified PEG-PLA micelles for targeted delivery to brain-derived endothelial cells in vitro. *J Microencapsul* 30, 701–708. doi:10.3109/02652048.2013.778907
- Mu, L.-M., Bu, Y.-Z., Liu, L., Xie, H.-J., Ju, R.-J., Wu, J.-S., Zeng, F., Zhao, Y., Zhang, J.-Y., Lu, W.-L., 2017. Lipid vesicles containing transferrin receptor binding peptide TfR-T12 and octa-arginine conjugate stearyl-R8 efficiently treat brain glioma along with glioma stem cells. *Sci Rep* 7, 3487. doi:10.1038/s41598-017-03805-7
- Muldoon, L.L., Pagel, M.A., Kroll, R.A., Roman-Goldstein, S., Jones, R.S., Neuwelt, E.A., 1999. A physiological barrier distal to the anatomic blood-brain barrier in a model of transvascular delivery. *AJNR Am J Neuroradiol* 20, 217–222.
- Muldoon, L.L., Varallyay, P., Kraemer, D.F., Kiwic, G., Pinkston, K., Walker-Rosenfeld, S.L., Neuwelt, E.A., 2004. Trafficking of superparamagnetic iron oxide particles (Combidex) from brain to lymph nodes in the rat. *Neuropathol. Appl. Neurobiol.* 30, 70–79.
- Murray, C.J.L., Vos, T., Lozano, R., Naghavi, M., Flaxman, A.D., Michaud, C., Ezzati, M., Shibuya, K., Salomon, J.A., Abdalla, S., Aboyans, V., Abraham, J., Ackerman, I., Aggarwal, R., Ahn, S.Y., Ali, M.K., Alvarado, M., Anderson, H.R., Anderson, L.M., Andrews, K.G., Atkinson, C., Baddour, L.M., Bahalim, A.N., Barker-Collo, S., Barrero, L.H., Bartels, D.H., Basáñez, M.-G., Baxter, A., Bell, M.L., Benjamin, E.J., Bennett, D., Bernabé, E., Bhalla, K., Bhandari, B., Bikbov, B., Bin Abdulhak, A., Birbeck, G., Black, J.A., Blencowe, H., Blore, J.D., Blyth, F., Bolliger, I., Bonaventure, A., Boufous, S., Bourne, R., Boussinesq, M., Braithwaite, T., Brayne, C., Bridgett, L., Brooker, S., Brooks, P., Brugha, T.S., Bryan-Hancock, C., Bucello, C., Buchbinder, R., Buckle, G., Budke, C.M., Burch, M., Burney, P., Burstein, R., Calabria, B., Campbell, B., Canter, C.E., Carabin, H., Carapetis, J., Carmona, L., Cella, C., Charlson, F., Chen, H., Cheng, A.T.-A., Chou, D., Chugh, S.S., Coffeng, L.E., Colan, S.D., Colquhoun, S., Colson, K.E., Condon, J., Connor, M.D., Cooper, L.T., Corriere, M., Cortinovis, M., de Vaccaro, K.C., Couser, W., Cowie, B.C., Criqui, M.H., Cross, M., Dabhadkar, K.C., Dahiya, M., Dahodwala, N., Damsere-Derry, J., Danaei, G., Davis, A., De Leo, D., Degenhardt, L., Dellavalle, R., Delossantos, A., Denenberg, J., Derrett, S., Jarlais, Des, D.C., Dharmaratne, S.D., Dherani, M., Diaz-Torne, C., Dolk, H., Dorsey, E.R., Driscoll, T., Duber, H., Ebel, B., Edmond, K., Elbaz, A., Ali, S.E., Erskine, H., Erwin, P.J., Espindola, P., Ewoigbokhan, S.E., Farzadfar, F., Feigin, V., Felson, D.T., Ferrari, A., Ferri, C.P., Fèvre, E.M., Finucane, M.M., Flaxman, S., Flood, L., Foreman, K., Forouzanfar, M.H., Fowkes, F.G.R., Fransen, M., Freeman, M.K., Gabbe, B.J., Gabriel, S.E., Gakidou, E., Ganatra, H.A., Garcia, B., Gaspari, F., Gillum, R.F., Gmel, G., Gonzalez-Medina, D., Gosselin, R., Grainger, R., Grant, B., Groeger, J., Guillemin, F., Gunnell, D.,

Gupta, R., Haagsma, J., Hagan, H., Halasa, Y.A., Hall, W., Haring, D., Haro, J.M., Harrison, J.E., Havmoeller, R., Hay, R.J., Higashi, H., Hill, C., Hoen, B., Hoffman, H., Hotez, P.J., Hoy, D., Huang, J.J., Ibeanusi, S.E., Jacobsen, K.H., James, S.L., Jarvis, D., Jassrasaria, R., Jayaraman, S., Johns, N., Jonas, J.B., Karthikeyan, G., Kassebaum, N., Kawakami, N., Keren, A., Khoo, J.-P., King, C.H., Knowlton, L.M., Kobusingye, O., Koranteng, A., Krishnamurthi, R., Laden, F., Lalloo, R., Laslett, L.L., Lathlean, T., Leasher, J.L., Lee, Y.Y., Leigh, J., Levinson, D., Lim, S.S., Limb, E., Lin, J.K., Lipnick, M., Lipshultz, S.E., Liu, W., Loane, M., Ohno, S.L., Lyons, R., Mabweijano, J., MacIntyre, M.F., Malekzadeh, R., Mallinger, L., Manivannan, S., Marcenes, W., March, L., Margolis, D.J., Marks, G.B., Marks, R., Matsumori, A., Matzopoulos, R., Mayosi, B.M., McAnulty, J.H., McDermott, M.M., McGill, N., McGrath, J., Medina-Mora, M.E., Meltzer, M., Mensah, G.A., Merriman, T.R., Meyer, A.-C., Miglioli, V., Miller, M., Miller, T.R., Mitchell, P.B., Mock, C., Mocumbi, A.O., Moffitt, T.E., Mokdad, A.A., Monasta, L., Montico, M., Moradi-Lakeh, M., Moran, A., Morawska, L., Mori, R., Murdoch, M.E., Mwaniki, M.K., Naidoo, K., Nair, M.N., Naldi, L., Narayan, K.M.V., Nelson, P.K., Nelson, R.G., Nevitt, M.C., Newton, C.R., Nolte, S., Norman, P., Norman, R., O'Donnell, M., O'Hanlon, S., Olives, C., Omer, S.B., Ortblad, K., Osborne, R., Ozgediz, D., Page, A., Pahari, B., Pandian, J.D., Rivero, A.P., Patten, S.B., Pearce, N., Padilla, R.P., Perez-Ruiz, F., Perico, N., Pesudovs, K., Phillips, D., Phillips, M.R., Pierce, K., Pion, S., Polanczyk, G.V., Polinder, S., Pope, C.A., Popova, S., Porrini, E., Pourmalek, F., Prince, M., Pullan, R.L., Ramaiah, K.D., Ranganathan, D., Razavi, H., Regan, M., Rehm, J.T., Rein, D.B., Remuzzi, G., Richardson, K., Rivara, F.P., Roberts, T., Robinson, C., De Leòn, F.R., Ronfani, L., Room, R., Rosenfeld, L.C., Rushton, L., Sacco, R.L., Saha, S., Sampson, U., Sanchez-Riera, L., Sanman, E., Schwebel, D.C., Scott, J.G., Segui-Gomez, M., Shahraz, S., Shepard, D.S., Shin, H., Shivakoti, R., Singh, D., Singh, G.M., Singh, J.A., Singleton, J., Sleet, D.A., Sliwa, K., Smith, E., Smith, J.L., Stapelberg, N.J.C., Steer, A., Steiner, T., Stolk, W.A., Stovner, L.J., Sudfeld, C., Syed, S., Tamburlini, G., Tavakkoli, M., Taylor, H.R., Taylor, J.A., Taylor, W.J., Thomas, B., Thomson, W.M., Thurston, G.D., Tleyjeh, I.M., Tonelli, M., Towbin, J.A., Truelsen, T., Tsilimbaris, M.K., Ubeda, C., Undurraga, E.A., van der Werf, M.J., van Os, J., Vavilala, M.S., Venketasubramanian, N., Wang, M., Wang, W., Watt, K., Weatherall, D.J., Weinstock, M.A., Weintraub, R., Weisskopf, M.G., Weissman, M.M., White, R.A., Whiteford, H., Wiebe, N., Wiersma, S.T., Wilkinson, J.D., Williams, H.C., Williams, S.R.M., Witt, E., Wolfe, F., Woolf, A.D., Wulf, S., Yeh, P.-H., Zaidi, A.K.M., Zheng, Z.-J., Zonies, D., Lopez, A.D., AlMazroa, M.A., Memish, Z.A., 2012. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380, 2197–2223. doi:10.1016/S0140-6736(12)61689-4

Münter, R., Kristensen, K., Pedersbæk, D., Larsen, J.B., Simonsen, J.B., Andresen, T.L., 2018. Dissociation of fluorescently labeled lipids from liposomes in biological environments challenges the interpretation of uptake studies. *Nanoscale* 6, 286–22724. doi:10.1039/c8nr07755j

- Nakano, R., Takagi-Maeda, S., Ito, Y., Kishimoto, S., Osato, T., Noguchi, K., Kurihara-Suda, K., Takahashi, N., 2019. A new technology for increasing therapeutic protein levels in the brain over extended periods. *PLoS ONE* 14, e0214404. doi:10.1371/journal.pone.0214404
- Nance, E., Timbie, K., Miller, G.W., Song, J., Louttit, C., Klivanov, A.L., Shih, T.-Y., Swaminathan, G., Tamargo, R.J., Woodworth, G.F., Hanes, J., Price, R.J., 2014a. Non-invasive delivery of stealth, brain-penetrating nanoparticles across the blood-brain barrier using MRI-guided focused ultrasound. *J Control Release* 189, 123–132. doi:10.1016/j.jconrel.2014.06.031
- Nance, E., Zhang, C., Shih, T.-Y., Xu, Q., Schuster, B.S., Hanes, J., 2014b. Brain-penetrating nanoparticles improve paclitaxel efficacy in malignant glioma following local administration. *ACS Nano* 8, 10655–10664. doi:10.1021/nm504210g
- Nance, E.A., Woodworth, G.F., Sailor, K.A., Shih, T.-Y., Xu, Q., Swaminathan, G., Xiang, D., Eberhart, C., Hanes, J., 2012. A dense poly(ethylene glycol) coating improves penetration of large polymeric nanoparticles within brain tissue. *Sci Transl Med* 4, 149ra119–149ra119. doi:10.1126/scitranslmed.3003594
- Naz, N., Malik, I.A., Sheikh, N., Ahmad, S., Khan, S., Blaschke, M., Schultze, F., Ramadori, G., 2012. Ferroportin-1 is a “nuclear-”negative acute-phase protein in rat liver: a comparison with other iron-transport proteins. *Lab. Invest.* 92, 842–856. doi:10.1038/labinvest.2012.52
- Niewoehner, J., Bohrmann, B., Collin, L., Urich, E., Sade, H., Maier, P., Rueger, P., Stracke, J.O., Lau, W., Tissot, A.C., Loetscher, H., Ghosh, A., Freskgård, P.-O., 2014. Increased brain penetration and potency of a therapeutic antibody using a monovalent molecular shuttle. *Neuron* 81, 49–60. doi:10.1016/j.neuron.2013.10.061
- Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M., Tsukita, S., 2003. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J. Cell Biol.* 161, 653–660. doi:10.1083/jcb.200302070
- Okuyama, T., Eto, Y., Sakai, N., Minami, K., Yamamoto, T., Sonoda, H., Yamaoka, M., Tachibana, K., Hirato, T., Sato, Y., 2019. Iduronate-2-Sulfatase with Anti-human Transferrin Receptor Antibody for Neuropathic Mucopolysaccharidosis II: A Phase 1/2 Trial. *Mol Ther* 27, 456–464. doi:10.1016/j.ymthe.2018.12.005
- Omori, N., Maruyama, K., Jin, G., Li, F., Wang, S.J., Hamakawa, Y., Sato, K., Nagano, I., Shoji, M., Abe, K., 2003. Targeting of post-ischemic cerebral endothelium in rat by liposomes bearing polyethylene glycol-coupled transferrin. *Neurol. Res.* 25, 275–279. doi:10.1179/016164103101201508
- Osborn, M.J., McElmurry, R.T., Peacock, B., Tolar, J., Blazar, B.R., 2008. Targeting of the CNS in MPS-IH using a nonviral transferrin- $\alpha$ -L-iduronidase fusion gene product. *Mol Ther* 16, 1459–1466. doi:10.1038/mt.2008.119
- Ostrowitzki, S., Lasser, R.A., Dorflinger, E., Scheltens, P., Barkhof, F., Nikolcheva, T., Ashford, E., Retout, S., Hofmann, C., Delmar, P., Klein, G., Andjelkovic, M., Dubois, B., Boada, M., Blennow, K., Santarelli,

- L., Fontoura, P., SCarlet RoAD Investigators, 2017. A phase III randomized trial of gantenerumab in prodromal Alzheimer's disease. *Alzheimers Res Ther* 9, 95. doi:10.1186/s13195-017-0318-y
- Pang, Z., Lu, W., Gao, H., Hu, K., Chen, J., Zhang, C., Gao, X., Jiang, X., Zhu, C., 2008. Preparation and brain delivery property of biodegradable polymersomes conjugated with OX26. *J Control Release* 128, 120–127. doi:10.1016/j.jconrel.2008.03.007
- Papademetriou, I.T., Garnacho, C., Schuchman, E.H., Muro, S., 2013. In vivo performance of polymer nanocarriers dually-targeted to epitopes of the same or different receptors. *Biomaterials* 34, 3459–3466. doi:10.1016/j.biomaterials.2013.01.069
- Papadia, K., Giannou, A.D., Markoutsas, E., Bigot, C., Vanhoute, G., Mourtas, S., Van der Linded, A., Stathopoulos, G.T., Antimisiaris, S.G., 2017. Multifunctional LUV liposomes decorated for BBB and amyloid targeting - B. In vivo brain targeting potential in wild-type and APP/PS1 mice. *Eur J Pharm Sci* 102, 180–187. doi:10.1016/j.ejps.2017.03.010
- Pardridge, W.M., 2014. Blood-brain barrier drug delivery of IgG fusion proteins with a transferrin receptor monoclonal antibody. *Expert Opin Drug Deliv* 12, 1–16. doi:10.1517/17425247.2014.952627
- Pardridge, W.M., 2005. Tyrosine hydroxylase replacement in experimental Parkinson's disease with transvascular gene therapy. *NeuroRx* 2, 129–138. doi:10.1602/neurorx.2.1.129
- Pardridge, W.M., Boado, R.J., Giugliani, R., Schmidt, M., 2018a. Plasma Pharmacokinetics of Valanafusp Alpha, a Human Insulin Receptor Antibody-Iduronidase Fusion Protein, in Patients with Mucopolysaccharidosis Type I. *BioDrugs* 32, 169–176. doi:10.1007/s40259-018-0264-7
- Pardridge, W.M., Boado, R.J., Kang, Y.S., 1995. Vector-mediated delivery of a polyamide (“peptide”) nucleic acid analogue through the blood-brain barrier in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 92, 5592–5596.
- Pardridge, W.M., Boado, R.J., Patrick, D.J., Ka-Wai Hui, E., Lu, J.Z., 2018b. Blood-Brain Barrier Transport, Plasma Pharmacokinetics, and Neuropathology Following Chronic Treatment of the Rhesus Monkey with a Brain Penetrating Humanized Monoclonal Antibody Against the Human Transferrin Receptor. *Mol. Pharm.* 15, 5207–5216. doi:10.1021/acs.molpharmaceut.8b00730
- Pardridge, W.M., Buciak, J.L., Friden, P.M., 1991. Selective transport of an anti-transferrin receptor antibody through the blood-brain barrier in vivo. *J. Pharmacol. Exp. Ther.* 259, 66–70.
- Pardridge, W.M., Kang, Y.S., Buciak, J.L., 1994. Transport of human recombinant brain-derived neurotrophic factor (BDNF) through the rat blood-brain barrier in vivo using vector-mediated peptide drug delivery. *Pharm. Res.* 11, 738–746.
- Pardridge, W.M., Wu, D., Sakane, T., 1998. Combined use of carboxyl-directed protein pegylation and vector-mediated blood-brain barrier drug delivery system optimizes brain uptake of brain-derived neurotrophic factor following intravenous administration. *Pharm. Res.* 15, 576–582.

- Paris-Robidas, S., Brouard, D., Emond, V., Parent, M., Calon, F., 2016. Internalization of targeted quantum dots by brain capillary endothelial cells in vivo. *J. Cereb. Blood Flow Metab.* 36, 731–742. doi:10.1177/0271678X15608201
- Paris-Robidas, S., Emond, V., Tremblay, C., Soulet, D., Calon, F., 2011. In vivo labeling of brain capillary endothelial cells after intravenous injection of monoclonal antibodies targeting the transferrin receptor. *Mol. Pharmacol.* 80, 32–39. doi:10.1124/mol.111.071027
- Park, K., 2017. The drug delivery field at the inflection point: Time to fight its way out of the egg. *J Control Release* 267, 2–14. doi:10.1016/j.jconrel.2017.07.030
- Patel, A., Toia, G.V., Colletta, K., Bradaric, B.D., Carvey, P.M., Hendey, B., 2011. An angiogenic inhibitor, cyclic RGDfV, attenuates MPTP-induced dopamine neuron toxicity. *Exp. Neurol.* 231, 160–170. doi:10.1016/j.expneurol.2011.06.004
- Patel, B.N., David, S., 1997. A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. *J. Biol. Chem.* 272, 20185–20190.
- Patil, R., Portilla-Arias, J., Ding, H., Inoue, S., Konda, B., Hu, J., Wawrowsky, K.A., Shin, P.K., Black, K.L., Holler, E., Ljubimova, J.Y., 2010. Temozolomide delivery to tumor cells by a multifunctional nano vehicle based on poly( $\beta$ -L-malic acid). *Pharm. Res.* 27, 2317–2329. doi:10.1007/s11095-010-0091-0
- Pizzo, M.E., Wolak, D.J., Kumar, N.N., Brunette, E., Brunquell, C.L., Hannocks, M.-J., Abbott, N.J., Meyerand, M.E., Sorokin, L., Stanimirovic, D.B., Thorne, R.G., 2018. Intrathecal antibody distribution in the rat brain: surface diffusion, perivascular transport and osmotic enhancement of delivery. *J. Physiol. (Lond.)* 596, 445–475. doi:10.1113/JP275105
- Plog, B.A., Mestre, H., Olveda, G.E., Sweeney, A.M., Kenney, H.M., Cove, A., Dholakia, K.Y., Tithof, J., Nevins, T.D., Lundgaard, I., Du, T., Kelley, D.H., Nedergaard, M., 2018. Transcranial optical imaging reveals a pathway for optimizing the delivery of immunotherapeutics to the brain. *JCI Insight* 3, 1188. doi:10.1172/jci.insight.120922
- Prabhakar, K., Afzal, S.M., Kumar, P.U., Rajanna, A., Kishan, V., 2011. Brain delivery of transferrin coupled indinavir submicron lipid emulsions--pharmacokinetics and tissue distribution. *Colloids Surf B Biointerfaces* 86, 305–313. doi:10.1016/j.colsurfb.2011.04.013
- Prades, R., Guerrero, S., Araya, E., Molina, C., Salas, E., Zurita, E., Selva, J., Egea, G., López-Iglesias, C., Teixidó, M., Kogan, M.J., Giralt, E., 2012. Delivery of gold nanoparticles to the brain by conjugation with a peptide that recognizes the transferrin receptor. *Biomaterials* 33, 7194–7205. doi:10.1016/j.biomaterials.2012.06.063
- Preston, J.E., Joan Abbott, N., Begley, D.J., 2014. Transcytosis of macromolecules at the blood-brain barrier. *Adv. Pharmacol.* 71, 147–163. doi:10.1016/bs.apha.2014.06.001
- R'zik, S., Loo, M., Beguin, Y., 2001. Reticulocyte transferrin receptor (TfR) expression and contribution to soluble TfR levels. *Haematologica* 86, 244–251.

- Ramalho, M.J., Sevin, E., Gosselet, F., Lima, J., Coelho, M.A.N., Loureiro, J.A., Pereira, M.C., 2018. Receptor-mediated PLGA nanoparticles for glioblastoma multiforme treatment. *Int J Pharm* 545, 84–92. doi:10.1016/j.ijpharm.2018.04.062
- Reth, M., 2013. Matching cellular dimensions with molecular sizes. *Nat. Immunol.* 14, 765–767. doi:10.1038/ni.2621
- Roberts, R.L., Fine, R.E., Sandra, A., 1993. Receptor-mediated endocytosis of transferrin at the blood-brain barrier. *J. Cell. Sci.* 104 ( Pt 2), 521–532.
- Roche, 2019. Roche to discontinue Phase III CREAD 1 and 2 clinical studies of crenezumab in early Alzheimer's disease (AD) [WWW Document]. Roche Press Releases. URL <https://www.roche.com/media/releases/med-cor-2019-01-30.htm> (accessed 2.26.19).
- Ruderisch, N., Schlatter, D., Kuglstatter, A., Guba, W., Huber, S., Cusulin, C., Benz, J., Rufer, A.C., Hoernschemeyer, J., Schweitzer, C., Bülau, T., Gärtner, A., Hoffmann, E., Niewoehner, J., Patsch, C., Baumann, K., Loetscher, H., KITAS, E., Freskgård, P.-O., 2017. Potent and Selective BACE-1 Peptide Inhibitors Lower Brain A $\beta$  Levels Mediated by Brain Shuttle Transport. *EBioMedicine* 24, 76–92. doi:10.1016/j.ebiom.2017.09.004
- Sade, H., Baumgartner, C., Hugenmatter, A., Moessner, E., Freskgård, P.-O., Niewoehner, J., 2014. A human blood-brain barrier transcytosis assay reveals antibody transcytosis influenced by pH-dependent receptor binding. *PLoS ONE* 9, e96340. doi:10.1371/journal.pone.0096340
- Sahin, A., Yoyen-Ermis, D., Caban-Toktas, S., Horzum, U., Aktaş, Y., Couvreur, P., Esendagli, G., Capan, Y., 2017. Evaluation of brain-targeted chitosan nanoparticles through blood-brain barrier cerebral microvessel endothelial cells. *J Microencapsul* 34, 659–666. doi:10.1080/02652048.2017.1375039
- Saito, R., Bringas, J.R., McKnight, T.R., Wendland, M.F., Mamot, C., Drummond, D.C., Kirpotin, D.B., Park, J.W., Berger, M.S., Bankiewicz, K.S., 2004. Distribution of liposomes into brain and rat brain tumor models by convection-enhanced delivery monitored with magnetic resonance imaging. *Cancer Research* 64, 2572–2579.
- Saito, R., Krauze, M.T., Noble, C.O., Drummond, D.C., Kirpotin, D.B., Berger, M.S., Park, J.W., Bankiewicz, K.S., 2006. Convection-enhanced delivery of Ls-TPT enables an effective, continuous, low-dose chemotherapy against malignant glioma xenograft model. *Neuro-oncology* 8, 205–214. doi:10.1215/15228517-2006-001
- Saito, Y., Buciak, J., Yang, J., Pardridge, W.M., 1995. Vector-mediated delivery of 125I-labeled beta-amyloid peptide A beta 1-40 through the blood-brain barrier and binding to Alzheimer disease amyloid of the A beta 1-40/vector complex. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10227–10231.
- Saitou, M., Furuse, M., Sasaki, H., Schulzke, J.D., Fromm, M., Takano, H., Noda, T., Tsukita, S., 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol. Biol. Cell* 11, 4131–4142.

- Salegio, E.A., Streeter, H., Dube, N., Hadaczek, P., Samaranch, L., Kells, A.P., San Sebastian, W., Zhai, Y., Bringas, J., Xu, T., Forsayeth, J., Bankiewicz, K.S., 2014. Distribution of nanoparticles throughout the cerebral cortex of rodents and non-human primates: Implications for gene and drug therapy. *Front Neuroanat* 8, 9. doi:10.3389/fnana.2014.00009
- Salloway, S., Sperling, R., Brashear, H.R., 2014a. Phase 3 trials of solanezumab and bapineuzumab for Alzheimer's disease. *N Engl J Med* 370, 1460–1460. doi:10.1056/NEJMc1402193
- Salloway, S., Sperling, R., Fox, N.C., Blennow, K., Klunk, W., Raskind, M., Sabbagh, M., Honig, L.S., Porsteinsson, A.P., Ferris, S., Reichert, M., Ketter, N., Nejadnik, B., Guenzler, V., Miloslavsky, M., Wang, D., Lu, Y., Lull, J., Tudor, I.C., Liu, E., Grundman, M., Yuen, E., Black, R., Brashear, H.R., Bapineuzumab 301 and 302 Clinical Trial Investigators, 2014b. Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease. *N Engl J Med* 370, 322–333. doi:10.1056/NEJMoa1304839
- Salvati, E., Re, F., Sesana, S., Cambianica, I., Sancini, G., Masserini, M., Gregori, M., 2013. Liposomes functionalized to overcome the blood-brain barrier and to target amyloid- $\beta$  peptide: the chemical design affects the permeability across an in vitro model. *Int J Nanomedicine* 8, 1749–1758. doi:10.2147/IJN.S42783
- Salzano, G., Zappavigna, S., Luce, A., D'Onofrio, N., Balestrieri, M.L., Grimaldi, A., Lusa, S., Ingrosso, D., Artuso, S., Porru, M., Leonetti, C., Caraglia, M., De Rosa, G., 2016. Transferrin-Targeted Nanoparticles Containing Zoledronic Acid as a Potential Tool to Inhibit Glioblastoma Growth. *J Biomed Nanotechnol* 12, 811–830.
- Samaranch, L., Salegio, E.A., San Sebastian, W., Kells, A.P., Bringas, J.R., Forsayeth, J., Bankiewicz, K.S., 2013. Strong cortical and spinal cord transduction after AAV7 and AAV9 delivery into the cerebrospinal fluid of nonhuman primates. *Hum. Gene Ther.* 24, 526–532. doi:10.1089/hum.2013.005
- Santos, dos, W.L., Rahman, J., Klein, N., Male, D.K., 1995. Distribution and analysis of surface charge on brain endothelium in vitro and in situ. *Acta Neuropathol.* 90, 305–311.
- Satlin, A., Wang, J., Logovinsky, V., Berry, S., Swanson, C., Dhadda, S., Berry, D.A., 2016. Design of a Bayesian adaptive phase 2 proof-of-concept trial for BAN2401, a putative disease-modifying monoclonal antibody for the treatment of Alzheimer's disease. *Alzheimers Dement (N Y)* 2, 1–12. doi:10.1016/j.trci.2016.01.001
- Schmidt, J., Metselaar, J.M., Wauben, M.H.M., Toyka, K.V., Storm, G., Gold, R., 2003. Drug targeting by long-circulating liposomal glucocorticosteroids increases therapeutic efficacy in a model of multiple sclerosis. *Brain* 126, 1895–1904. doi:10.1093/brain/awg176
- Sehlin, D., Fang, X.T., Cato, L., Antoni, G., Lannfelt, L., Syvänen, S., 2016. Antibody-based PET imaging of amyloid beta in mouse models of Alzheimer's disease. *Nat Commun* 7, 10759. doi:10.1038/ncomms10759

- Sehlin, D., Fang, X.T., Meier, S.R., Jansson, M., Syvänen, S., 2017. Pharmacokinetics, biodistribution and brain retention of a bispecific antibody-based PET radioligand for imaging of amyloid- $\beta$ . *Sci Rep* 7, 17254. doi:10.1038/s41598-017-17358-2
- Semple, S.C., Akinc, A., Chen, J., Sandhu, A.P., Mui, B.L., Cho, C.K., Sah, D.W.Y., Stebbing, D., Crosley, E.J., Yaworski, E., Hafez, I.M., Dorkin, J.R., Qin, J., Lam, K., Rajeev, K.G., Wong, K.F., Jeffs, L.B., Nechev, L., Eisenhardt, M.L., Jayaraman, M., Kazem, M., Maier, M.A., Srinivasulu, M., Weinstein, M.J., Chen, Q., Alvarez, R., Barros, S.A., De, S., Klimuk, S.K., Borland, T., Kosovrasti, V., Cantley, W.L., Tam, Y.K., Manoharan, M., Ciufolini, M.A., Tracy, M.A., de Fougerolles, A., MacLachlan, I., Cullis, P.R., Madden, T.D., Hope, M.J., 2010. Rational design of cationic lipids for siRNA delivery. *Nat. Biotechnol.* 28, 172–176. doi:10.1038/nbt.1602
- Sengillo, J.D., Winkler, E.A., Walker, C.T., Sullivan, J.S., Johnson, M., Zlokovic, B.V., 2012. Deficiency in mural vascular cells Coincides with Blood-Brain Barrier Disruption in Alzheimer's Disease. *Brain Pathol.* doi:10.1111/bpa.12004
- Servick, K., 2019. Another major drug candidate targeting the brain plaques of Alzheimer's disease has failed. What's left? *Science.* doi:10.1126/science.aax4236
- Sevigny, J., Chiao, P., Bussière, T., Weinreb, P.H., Williams, L., Maier, M., Dunstan, R., Salloway, S., Chen, T., Ling, Y., O'Gorman, J., Qian, F., Arastu, M., Li, M., Chollate, S., Brennan, M.S., Quintero-Monzon, O., Scannevin, R.H., Arnold, H.M., Engber, T., Rhodes, K., Ferrero, J., Hang, Y., Mikulskis, A., Grimm, J., Hock, C., Nitsch, R.M., Sandrock, A., 2016. The antibody aducanumab reduces A $\beta$  plaques in Alzheimer's disease. *Nature* 537, 50–56. doi:10.1038/nature19323
- Shankar, G., Pendley, C., Stein, K.E., 2007. A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs. *Nat. Biotechnol.* 25, 555–561. doi:10.1038/nbt1303
- Sharma, G., Lakkadwala, S., Modgil, A., Singh, J., 2016. The Role of Cell-Penetrating Peptide and Transferrin on Enhanced Delivery of Drug to Brain. *Int J Mol Sci* 17, 806. doi:10.3390/ijms17060806
- Sharma, G., Modgil, A., Layek, B., Arora, K., Sun, C., Law, B., Singh, J., 2013. Cell penetrating peptide tethered bi-ligand liposomes for delivery to brain in vivo: Biodistribution and transfection. *J Control Release* 167, 1–10. doi:10.1016/j.jconrel.2013.01.016
- Sharma, G., Modgil, A., Sun, C., Singh, J., 2012. Grafting of cell-penetrating peptide to receptor-targeted liposomes improves their transfection efficiency and transport across blood-brain barrier model. *J Pharm Sci* 101, 2468–2478. doi:10.1002/jps.23152
- Sharma, G., Modgil, A., Zhong, T., Sun, C., Singh, J., 2014. Influence of short-chain cell-penetrating peptides on transport of Doxorubicin encapsulating receptor-targeted liposomes across brain endothelial barrier. *Pharm. Res.* 31, 1194–1209. doi:10.1007/s11095-013-1242-x

- Shaw, A., Hoffecker, I.T., Smyrlaki, I., Rosa, J., Grevys, A., Bratlie, D., Sandlie, I., Michaelsen, T.E., Andersen, J.T., Högberg, B., 2019. Binding to nanopatterned antigens is dominated by the spatial tolerance of antibodies. *Nat Nanotechnol* 14, 184–190. doi:10.1038/s41565-018-0336-3
- Shi, N., Boado, R.J., Pardridge, W.M., 2001a. Receptor-mediated gene targeting to tissues in vivo following intravenous administration of pegylated immunoliposomes. *Pharm. Res.* 18, 1091–1095.
- Shi, N., Pardridge, W.M., 2000. Noninvasive gene targeting to the brain. *Proc. Natl. Acad. Sci. U.S.A.* 97, 7567–7572. doi:10.1073/pnas.130187497
- Shi, N., Zhang, Y., Zhu, C., Boado, R.J., Pardridge, W.M., 2001b. Brain-specific expression of an exogenous gene after i.v. administration. *Proc. Natl. Acad. Sci. U.S.A.* 98, 12754–12759. doi:10.1073/pnas.221450098
- Shin, S.U., Friden, P., Moran, M., Olson, T., Kang, Y.S., Pardridge, W.M., Morrison, S.L., 1995. Transferrin-antibody fusion proteins are effective in brain targeting. *Proc. Natl. Acad. Sci. U.S.A.* 92, 2820–2824.
- Siddappa, A.J.M., Rao, R.B., Wobken, J.D., Leibold, E.A., Connor, J.R., Georgieff, M.K., 2002. Developmental changes in the expression of iron regulatory proteins and iron transport proteins in the perinatal rat brain. *J. Neurosci. Res.* 68, 761–775. doi:10.1002/jnr.10246
- Siemers, E.R., Sundell, K.L., Carlson, C., Case, M., Sethuraman, G., Liu-Seifert, H., Dowsett, S.A., Pontecorvo, M.J., Dean, R.A., Demattos, R., 2016. Phase 3 solanezumab trials: Secondary outcomes in mild Alzheimer's disease patients. *Alzheimers Dement* 12, 110–120. doi:10.1016/j.jalz.2015.06.1893
- Simpson, I.A., Ponnuru, P., Klinger, M.E., Myers, R.L., Devraj, K., Coe, C.L., Lubach, G.R., Carruthers, A., Connor, J.R., 2015. A novel model for brain iron uptake: introducing the concept of regulation. *J. Cereb. Blood Flow Metab.* 35, 48–57. doi:10.1038/jcbfm.2014.168
- Singh, R., Norret, M., House, M.J., Galabura, Y., Bradshaw, M., Ho, D., Woodward, R.C., St Pierre, T.G., Luzinov, I., Smith, N.M., Lim, L.Y., Iyer, K.S., 2016. Dose-Dependent Therapeutic Distinction between Active and Passive Targeting Revealed Using Transferrin-Coated PGMA Nanoparticles. *Small* 12, 351–359. doi:10.1002/smll.201502730
- Siupka, P., Hersom, M.N., Lykke-Hartmann, K., Johnsen, K.B., Thomsen, L.B., Andresen, T.L., Moos, T., Abbott, N.J., Brodin, B., Nielsen, M.S., 2017. Bidirectional apical-basal traffic of the cation-independent mannose-6-phosphate receptor in brain endothelial cells. *J. Cereb. Blood Flow Metab.* 37, 2598–2613. doi:10.1177/0271678X17700665
- Skinner, M.K., Cosand, W.L., Griswold, M.D., 1984. Purification and characterization of testicular transferrin secreted by rat Sertoli cells. *Biochem. J.* 218, 313–320.
- Skjørringe, T., Burkhart, A., Johnsen, K.B., Moos, T., 2015. Divalent metal transporter 1 (DMT1) in the brain: implications for a role in iron transport at the blood-brain barrier, and neuronal and glial pathology. *Front Mol Neurosci* 8, 19. doi:10.3389/fnmol.2015.00019

- Snipstad, S., Hak, S., Baghirov, H., Sulheim, E., Mørch, Ý., Lélú, S., Haartman, von, E., Bäck, M., Nilsson, K.P.R., Klymchenko, A.S., de Lange Davies, C., Åslund, A.K.O., 2016. Labeling nanoparticles: Dye leakage and altered cellular uptake. *Cytometry* 91, 760–766. doi:10.1002/cyto.a.22853
- Somani, S., Blatchford, D.R., Millington, O., Stevenson, M.L., Dufès, C., 2014. Transferrin-bearing polypropylenimine dendrimer for targeted gene delivery to the brain. *J Control Release* 188, 78–86. doi:10.1016/j.jconrel.2014.06.006
- Sonali, Agrawal, P., Singh, R.P., Rajesh, C.V., Singh, S., Vijayakumar, M.R., Pandey, B.L., Muthu, M.S., 2016a. Transferrin receptor-targeted vitamin E TPGS micelles for brain cancer therapy: preparation, characterization and brain distribution in rats. *Drug Deliv* 23, 1788–1798. doi:10.3109/10717544.2015.1094681
- Sonali, Singh, R.P., Singh, N., Sharma, G., Vijayakumar, M.R., Koch, B., Singh, S., Singh, U., Dash, D., Pandey, B.L., Muthu, M.S., 2016b. Transferrin liposomes of docetaxel for brain-targeted cancer applications: formulation and brain theranostics. *Drug Deliv* 23, 1261–1271. doi:10.3109/10717544.2016.1162878
- Song, B.-W., Vinters, H.V., Wu, D., Pardridge, W.M., 2002. Enhanced neuroprotective effects of basic fibroblast growth factor in regional brain ischemia after conjugation to a blood-brain barrier delivery vector. *J. Pharmacol. Exp. Ther.* 301, 605–610.
- Soni, V., Kohli, D.V., Jain, S.K., 2005. Transferrin coupled liposomes as drug delivery carriers for brain targeting of 5-fluorouracil. *J Drug Target* 13, 245–250. doi:10.1080/10611860500107401
- Sonoda, H., Morimoto, H., Yoden, E., Koshimura, Y., Kinoshita, M., Golovina, G., Takagi, H., Yamamoto, R., Minami, K., Mizoguchi, A., Tachibana, K., Hirato, T., Takahashi, K., 2018. A Blood-Brain-Barrier-Penetrating Anti-human Transferrin Receptor Antibody Fusion Protein for Neuronopathic Mucopolysaccharidosis II. *Mol Ther* 26, 1366–1374. doi:10.1016/j.ymthe.2018.02.032
- Sperling, R.A., Rivera Gil, P., Zhang, F., Zanella, M., Parak, W.J., 2008. Biological applications of gold nanoparticles. *Chem Soc Rev* 37, 1896–1908. doi:10.1039/b712170a
- Sriramoju, B., Neerati, P., Kanwar, R.K., Kanwar, J.R., 2015. Brain targeted PLGA nanocarriers alleviating amyloid-B expression and preserving basal survivin in degenerating mice model. *Biochim. Biophys. Acta* 1852, 2423–2431. doi:10.1016/j.bbadis.2015.08.015
- St-Amour, I., Paré, I., Alata, W., Coulombe, K., Ringuette-Goulet, C., Drouin-Ouellet, J., Vandal, M., Soulet, D., Bazin, R., Calon, F., 2013. Brain bioavailability of human intravenous immunoglobulin and its transport through the murine blood-brain barrier. *J. Cereb. Blood Flow Metab.* 33, 1983–1992. doi:10.1038/jcbfm.2013.160
- Staquicini, F.I., Ozawa, M.G., Moya, C.A., Driessen, W.H.P., Barbu, E.M., Nishimori, H., Soghomonyan, S., Flores, L.G., Liang, X., Paolillo, V., Alauddin, M.M., Basilion, J.P., Furnari, F.B., Bogler, O., Lang, F.F., Aldape, K.D., Fuller, G.N., Höök, M., Gelovani, J.G., Sidman, R.L., Cavenee, W.K., Pasqualini, R., Arap,

- W., 2011. Systemic combinatorial peptide selection yields a non-canonical iron-mimicry mechanism for targeting tumors in a mouse model of human glioblastoma. *J. Clin. Invest.* 121, 161–173. doi:10.1172/JCI44798
- Steere, A.N., Chasteen, N.D., Miller, B.F., Smith, V.C., MacGillivray, R.T.A., Mason, A.B., 2012. Structure-based mutagenesis reveals critical residues in the transferrin receptor participating in the mechanism of pH-induced release of iron from human serum transferrin. *Biochemistry* 51, 2113–2121. doi:10.1021/bi3001038
- Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med.* 18, 321–336.
- Suk, J.S., Xu, Q., Kim, N., Hanes, J., Ensign, L.M., 2016. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Adv. Drug Deliv. Rev.* 99, 28–51. doi:10.1016/j.addr.2015.09.012
- Sulheim, E., Mørch, Ý., Snipstad, S., Borgos, S.E., Miletic, H., Bjerkgvig, R., Davies, C. de L., Åslund, A.K.O., 2019. Therapeutic Effect of Cabazitaxel and Blood-Brain Barrier opening in a Patient-Derived Glioblastoma Model. *Nanotheranostics* 3, 103–112. doi:10.7150/ntno.31479
- Sumbria, R.K., Boado, R.J., Pardridge, W.M., 2013a. Combination stroke therapy in the mouse with blood-brain barrier penetrating IgG-GDNF and IgG-TNF decoy receptor fusion proteins. *Brain Research* 1507, 91–96. doi:10.1016/j.brainres.2013.02.022
- Sumbria, R.K., Zhou, Q.-H., Hui, E.K.-W., Lu, J.Z., Boado, R.J., Pardridge, W.M., 2013b. Pharmacokinetics and brain uptake of an IgG-TNF decoy receptor fusion protein following intravenous, intraperitoneal, and subcutaneous administration in mice. *Mol. Pharm.* 10, 1425–1431. doi:10.1021/mp400004a
- Sun, H., Li, H., Sadler, P.J., 1999. Transferrin as a metal ion mediator. *Chem. Rev.* 99, 2817–2842.
- Sun, J., Boado, R.J., Pardridge, W.M., Sumbria, R.K., 2019. Plasma pharmacokinetics of high-affinity transferrin receptor antibody-erythropoietin fusion protein is a function of effector attenuation in mice. *Mol. Pharm.* acs.molpharmaceut.9b00369. doi:10.1021/acs.molpharmaceut.9b00369
- Sun, T., Wu, H., Li, Y., Huang, Y., Yao, L., Chen, X., Han, X., Zhou, Y., Du, Z., 2017. Targeting transferrin receptor delivery of temozolomide for a potential glioma stem cell-mediated therapy. *Oncotarget* 8, 74451–74465. doi:10.18632/oncotarget.20165
- Suresh Reddy, J., Venkateswarlu, V., Koning, G.A., 2006. Radioprotective effect of transferrin targeted citicoline liposomes. *J Drug Target* 14, 13–19. doi:10.1080/10611860600613241
- Sweeney, M.D., Sagare, A.P., Zlokovic, B.V., 2018. Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nat Rev Neurol* 14, 133–150. doi:10.1038/nrneurol.2017.188
- Sylvester, S.R., Griswold, M.D., 1984. Localization of transferrin and transferrin receptors in rat testes. *Biol. Reprod.* 31, 195–203.

- Syvänen, S., Fang, X.T., Hultqvist, G., Meier, S.R., Lannfelt, L., Sehlin, D., 2017. A bispecific Tribody PET radioligand for visualization of amyloid-beta protofibrils - a new concept for neuroimaging. *Neuroimage* 148, 55–63. doi:10.1016/j.neuroimage.2017.01.004
- Syvänen, S., Hultqvist, G., Gustavsson, T., Gumucio, A., Laudon, H., Söderberg, L., Ingelsson, M., Lannfelt, L., Sehlin, D., 2018. Efficient clearance of A $\beta$  protofibrils in A $\beta$ PP-transgenic mice treated with a brain-penetrating bifunctional antibody. *Alzheimers Res Ther* 10, 49. doi:10.1186/s13195-018-0377-8
- Tanaka, N., Kida, S., Kinoshita, M., Morimoto, H., Shibasaki, T., Tachibana, K., Yamamoto, R., 2018. Evaluation of cerebrospinal fluid heparan sulfate as a biomarker of neuropathology in a murine model of mucopolysaccharidosis type II using high-sensitivity LC/MS/MS. *Mol. Genet. Metab.* 125, 53–58. doi:10.1016/j.ymgme.2018.07.013
- Tang, X., Liang, Y., Zhu, Y., Xie, C., Yao, A., Chen, L., Jiang, Q., Liu, T., Wang, X., Qian, Y., Wei, J., Ni, W., Dai, J., Jiang, Z., Hou, W., 2015. Anti-transferrin receptor-modified amphotericin B-loaded PLA-PEG nanoparticles cure Candidal meningitis and reduce drug toxicity. *Int J Nanomedicine* 10, 6227–6241. doi:10.2147/IJN.S84656
- Taylor, E.M., Crowe, A., Morgan, E.H., 1991. Transferrin and iron uptake by the brain: effects of altered iron status. *J. Neurochem.* 57, 1584–1592. doi:10.1111/j.1471-4159.1991.tb06355.x
- Theil, E.C., 1987. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu. Rev. Biochem.* 56, 289–315. doi:10.1146/annurev.bi.56.070187.001445
- Thom, G., Burrell, M., Haqqani, A.S., Yogi, A., Lessard, E., Brunette, E., Delaney, C., Baumann, E., Callaghan, D., Rodrigo, N., Webster, C.I., Stanimirovic, D.B., 2018. Enhanced Delivery of Galanin Conjugates to the Brain through Bioengineering of the Anti-Transferrin Receptor Antibody OX26. *Mol. Pharm.* 15, 1420–1431. doi:10.1021/acs.molpharmaceut.7b00937
- Thorne, R.G., Nicholson, C., 2006. In vivo diffusion analysis with quantum dots and dextrans predicts the width of brain extracellular space. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5567–5572. doi:10.1073/pnas.0509425103
- Tillotson, B.J., Goulatis, L.I., Parenti, I., Duxbury, E., Shusta, E.V., 2015. Engineering an Anti-Transferrin Receptor ScFv for pH-Sensitive Binding Leads to Increased Intracellular Accumulation. *PLoS ONE* 10, e0145820. doi:10.1371/journal.pone.0145820
- Torchilin, V.P., 2014. Multifunctional, stimuli-sensitive nanoparticulate systems for drug delivery. *Nat Rev Drug Discov* 13, 813–827. doi:10.1038/nrd4333
- Tortorella, S., Karagiannis, T.C., 2014. Transferrin receptor-mediated endocytosis: a useful target for cancer therapy. *J. Membr. Biol.* 247, 291–307. doi:10.1007/s00232-014-9637-0
- Trinder, D., Baker, E., 2003. Transferrin receptor 2: a new molecule in iron metabolism. *Int. J. Biochem. Cell Biol.* 35, 292–296.

- Tucker, S., Möller, C., Tegerstedt, K., Lord, A., Laudon, H., Sjödahl, J., Söderberg, L., Spens, E., Sahlin, C., Waara, E.R., Satlin, A., Gellerfors, P., Osswald, G., Lannfelt, L., 2015. The murine version of BAN2401 (mAb158) selectively reduces amyloid- $\beta$  protofibrils in brain and cerebrospinal fluid of tg-ArcSwe mice. *J Alzheimers Dis* 43, 575–588. doi:10.3233/JAD-140741
- Turjeman, K., Bavli, Y., Kizelsztejn, P., Schilt, Y., Allon, N., Katzir, T.B., Sasson, E., Raviv, U., Ovadia, H., Barenholz, Y., 2015. Nano-Drugs Based on Nano Sterically Stabilized Liposomes for the Treatment of Inflammatory Neurodegenerative Diseases. *PLoS ONE* 10, e0130442. doi:10.1371/journal.pone.0130442
- Ueda, F., Raja, K.B., Simpson, R.J., Trowbridge, I.S., Bradbury, M.W., 1993. Rate of  $^{59}\text{Fe}$  uptake into brain and cerebrospinal fluid and the influence thereon of antibodies against the transferrin receptor. *J. Neurochem.* 60, 106–113.
- Ulbrich, K., Hekmatara, T., Herbert, E., Kreuter, J., 2009. Transferrin- and transferrin-receptor-antibody-modified nanoparticles enable drug delivery across the blood-brain barrier (BBB). *Eur J Pharm Biopharm* 71, 251–256. doi:10.1016/j.ejpb.2008.08.021
- van Gelder, W., Cleton-Soeteman, M.I., Huijskes-Heins, M.I., van Run, P.R., van Eijk, H.G., 1997. Transcytosis of 6.6-nm gold-labeled transferrin: an ultrastructural study in cultured porcine blood-brain barrier endothelial cells. *Brain Research* 746, 105–116.
- van Gelder, W., Huijskes-Heins, M.I., van Dijk, J.P., Cleton-Soeteman, M.I., van Eijk, H.G., 1995. Quantification of different transferrin receptor pools in primary cultures of porcine blood-brain barrier endothelial cells. *J. Neurochem.* 64, 2708–2715.
- van Rooy, I., Cakir-Tascioglu, S., Hennink, W.E., Storm, G., Schiffelers, R.M., Mastrobattista, E., 2011a. In vivo methods to study uptake of nanoparticles into the brain. *Pharm. Res.* 28, 456–471. doi:10.1007/s11095-010-0291-7
- van Rooy, I., Mastrobattista, E., Storm, G., Hennink, W.E., Schiffelers, R.M., 2011b. Comparison of five different targeting ligands to enhance accumulation of liposomes into the brain. *J Control Release* 150, 30–36. doi:10.1016/j.jconrel.2010.11.014
- Villaseñor, R., Lampe, J., Schwaninger, M., Collin, L., 2018. Intracellular transport and regulation of transcytosis across the blood-brain barrier. *Cell. Mol. Life Sci.* 34, 207–12. doi:10.1007/s00018-018-2982-x
- Villaseñor, R., Ozmen, L., Messaddeq, N., Grüniger, F., Loetscher, H., Keller, A., Betsholtz, C., Freskgård, P.-O., Collin, L., 2016. Trafficking of Endogenous Immunoglobulins by Endothelial Cells at the Blood-Brain Barrier. *Sci Rep* 6, 25658. doi:10.1038/srep25658
- Villaseñor, R., Schilling, M., Sundaresan, J., Lutz, Y., Collin, L., 2017. Sorting Tubules Regulate Blood-Brain Barrier Transcytosis. *Cell Rep* 21, 3256–3270. doi:10.1016/j.celrep.2017.11.055

- Visser, C.C., Stevanović, S., Heleen Voorwinden, L., Gaillard, P.J., Crommelin, D.J.A., Danhof, M., de Boer, A.G., 2004a. Validation of the transferrin receptor for drug targeting to brain capillary endothelial cells in vitro. *J Drug Target* 12, 145–150. doi:10.1080/10611860410001701706
- Visser, C.C., Stevanović, S., Voorwinden, L.H., van Bloois, L., Gaillard, P.J., Danhof, M., Crommelin, D.J.A., de Boer, A.G., 2005. Targeting liposomes with protein drugs to the blood-brain barrier in vitro. *Eur J Pharm Sci* 25, 299–305. doi:10.1016/j.ejps.2005.03.008
- Visser, C.C., Voorwinden, L.H., Crommelin, D.J.A., Danhof, M., de Boer, A.G., 2004b. Characterization and modulation of the transferrin receptor on brain capillary endothelial cells. *Pharm. Res.* 21, 761–769.
- Walus, L.R., Pardridge, W.M., Starzyk, R.M., Friden, P.M., 1996. Enhanced uptake of rsCD4 across the rodent and primate blood-brain barrier after conjugation to anti-transferrin receptor antibodies. *J. Pharmacol. Exp. Ther.* 277, 1067–1075.
- Wang, F., Bai, H.-R., Wang, J., Bai, Y.-Z., Dou, C.-W., 2011. Glioma growth inhibition in vitro and in vivo by single chain variable fragments of the transferrin receptor conjugated to survivin small interfering RNA. *J. Int. Med. Res.* 39, 1701–1712. doi:10.1177/147323001103900512
- Wang, Z., Zhao, Y., Jiang, Y., Lv, W., Wu, L., Wang, B., Lv, L., Xu, Q., Xin, H., 2015. Enhanced anti-ischemic stroke of ZL006 by T7-conjugated PEGylated liposomes drug delivery system. *Sci Rep* 5, 12651. doi:10.1038/srep12651
- Wardman, P., Candeias, L.P., 1996. Fenton Chemistry: An Introduction. *Radiation Research* 145, 523. doi:10.2307/3579270
- Watts, R.J., Dennis, M.S., 2013. Bispecific antibodies for delivery into the brain. *Curr Opin Chem Biol* 17, 393–399. doi:10.1016/j.cbpa.2013.03.023
- Weaver, M., Laske, D.W., 2003. Transferrin receptor ligand-targeted toxin conjugate (Tf-CRM107) for therapy of malignant gliomas. *J Neurooncol* 65, 3–13.
- Weber, F., Bohrmann, B., Niewoehner, J., Fischer, J.A.A., Rueger, P., Tiefenthaler, G., Moelleken, J., Bujotzek, A., Brady, K., Singer, T., Ebeling, M., Iglesias, A., Freskård, P.-O., 2018a. Brain Shuttle Antibody for Alzheimer's Disease with Attenuated Peripheral Effector Function due to an Inverted Binding Mode. *Cell Rep* 22, 149–162. doi:10.1016/j.celrep.2017.12.019
- Weber, F., Breustedt, D., Schlicht, S., Meyer, C.A., Niewoehner, J., Ebeling, M., Freskård, P.-O., Bruenker, P., Singer, T., Reth, M., Iglesias, A., 2018b. First Infusion Reactions are Mediated by FcγRIIIb and Neutrophils. *Pharm. Res.* 35, 169. doi:10.1007/s11095-018-2448-8
- Webster, C.I., Hatcher, J., Burrell, M., Thom, G., Thornton, P., Gurrell, I., Chessell, I., 2017. Enhanced delivery of IL-1 receptor antagonist to the central nervous system as a novel anti-transferrin receptor-IL-1RA fusion reverses neuropathic mechanical hypersensitivity. *Pain* 158, 660–668. doi:10.1097/j.pain.0000000000000810

- Whiteford, H.A., Ferrari, A.J., Degenhardt, L., Feigin, V., Vos, T., 2015. The global burden of mental, neurological and substance use disorders: an analysis from the Global Burden of Disease Study 2010. *PLoS ONE* 10, e0116820. doi:10.1371/journal.pone.0116820
- Wiley, D.T., Webster, P., Gale, A., Davis, M.E., 2013. Transcytosis and brain uptake of transferrin-containing nanoparticles by tuning avidity to transferrin receptor. *Proc. Natl. Acad. Sci. U.S.A.* 110, 8662–8667. doi:10.1073/pnas.1307152110
- Wolak, D.J., Pizzo, M.E., Thorne, R.G., 2015. Probing the extracellular diffusion of antibodies in brain using in vivo integrative optical imaging and ex vivo fluorescence imaging. *J Control Release* 197, 78–86. doi:10.1016/j.jconrel.2014.10.034
- Wolak, D.J., Thorne, R.G., 2013. Diffusion of macromolecules in the brain: implications for drug delivery. *Mol. Pharm.* 10, 1492–1504. doi:10.1021/mp300495e
- Wolburg, H., Lippoldt, A., 2002. Tight junctions of the blood-brain barrier: development, composition and regulation. *Vascul. Pharmacol.* 38, 323–337.
- Wolburg, H., Noell, S., Mack, A., Wolburg-Buchholz, K., Fallier-Becker, P., 2009. Brain endothelial cells and the glio-vascular complex. *Cell Tissue Res.* 335, 75–96. doi:10.1007/s00441-008-0658-9
- Wolburg, H., Wolburg-Buchholz, K., Kraus, J., Rascher-Eggstein, G., Liebner, S., Hamm, S., Duffner, F., Grote, E.-H., Risau, W., Engelhardt, B., 2003. Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme. *Acta Neuropathol.* 105, 586–592. doi:10.1007/s00401-003-0688-z
- Woodworth, G.F., Dunn, G.P., Nance, E.A., Hanes, J., Brem, H., 2014. Emerging insights into barriers to effective brain tumor therapeutics. *Front Oncol* 4, 126. doi:10.3389/fonc.2014.00126
- Wu, D., Boado, R.J., Pardridge, W.M., 1996. Pharmacokinetics and blood-brain barrier transport of [3H]-biotinylated phosphorothioate oligodeoxynucleotide conjugated to a vector-mediated drug delivery system. *J. Pharmacol. Exp. Ther.* 276, 206–211.
- Wu, D., Pardridge, W.M., 1999. Neuroprotection with noninvasive neurotrophin delivery to the brain. *Proc. Natl. Acad. Sci. U.S.A.* 96, 254–259.
- Wu, D., Pardridge, W.M., 1998. Pharmacokinetics and blood-brain barrier transport of an anti-transferrin receptor monoclonal antibody (OX26) in rats after chronic treatment with the antibody. *Drug Metab. Dispos.* 26, 937–939.
- Wu, D., Pardridge, W.M., 1996. Central nervous system pharmacologic effect in conscious rats after intravenous injection of a biotinylated vasoactive intestinal peptide analog coupled to a blood-brain barrier drug delivery system. *J. Pharmacol. Exp. Ther.* 279, 77–83.
- Wu, D., Song, B.-W., Vinters, H.V., Pardridge, W.M., 2002. Pharmacokinetics and brain uptake of biotinylated basic fibroblast growth factor conjugated to a blood-brain barrier drug delivery system. *J Drug Target* 10, 239–245. doi:10.1080/10611860290022679

- Wyatt, E.A., Davis, M.E., 2019. Method of establishing breast cancer brain metastases affects brain uptake and efficacy of targeted, therapeutic nanoparticles. *Bioeng Transl Med* 4, 30–37. doi:10.1002/btm2.10108
- Xia, C.-F., Zhang, Y., Zhang, Y., Boado, R.J., Pardridge, W.M., 2007. Intravenous siRNA of brain cancer with receptor targeting and avidin-biotin technology. *Pharm. Res.* 24, 2309–2316. doi:10.1007/s11095-007-9460-8
- Xie, L., Kang, H., Xu, Q., Chen, M.J., Liao, Y., Thiyagarajan, M., O'Donnell, J., Christensen, D.J., Nicholson, C., Iliff, J.J., Takano, T., Deane, R., Nedergaard, M., 2013. Sleep drives metabolite clearance from the adult brain. *Science* 342, 373–377. doi:10.1126/science.1241224
- Yadav, D.B., Maloney, J.A., Wildsmith, K.R., Fuji, R.N., Meilandt, W.J., Solanoy, H., Lu, Y., Peng, K., Wilson, B., Chan, P., Gadkar, K., Kosky, A., Goo, M., Daugherty, A., Couch, J.A., Keene, T., Hayes, K., Nikolas, L.J., Lane, D., Switzer, R., Adams, E., Watts, R.J., Scarce-Levie, K., Prabhu, S., Shafer, L., Thakker, D.R., Hildebrand, K., Atwal, J.K., 2017. Widespread brain distribution and activity following i.c.v. infusion of anti- $\beta$ -secretase (BACE1) in nonhuman primates. *Br. J. Pharmacol.* 174, 4173–4185. doi:10.1111/bph.14021
- Yan, F., Wang, Y., He, S., Ku, S., Gu, W., Ye, L., 2013. Transferrin-conjugated, fluorescein-loaded magnetic nanoparticles for targeted delivery across the blood-brain barrier. *J Mater Sci Mater Med* 24, 2371–2379. doi:10.1007/s10856-013-4993-3
- Yang, J., Fan, L., Wang, F., Luo, Y., Sui, X., Li, W., Zhang, X., Wang, Y., 2016. Rapid-releasing of HI-6 via brain-targeted mesoporous silica nanoparticles for nerve agent detoxification. *Nanoscale* 8, 9537–9547. doi:10.1039/c5nr06658a
- Yashunsky, V., Shimron, S., Lirtsman, V., Weiss, A.M., Melamed-Book, N., Golosovsky, M., Davidov, D., Aroeti, B., 2009. Real-time monitoring of transferrin-induced endocytic vesicle formation by mid-infrared surface plasmon resonance. *Biophys. J.* 97, 1003–1012. doi:10.1016/j.bpj.2009.05.052
- Yemisci, M., Caban, S., Fernandez-Megia, E., Capan, Y., Couvreur, P., Dalkara, T., 2018. Preparation and Characterization of Biocompatible Chitosan Nanoparticles for Targeted Brain Delivery of Peptides. *Methods Mol. Biol.* 1727, 443–454. doi:10.1007/978-1-4939-7571-6\_36
- Yemisci, M., Caban, S., Gürsoy-Özdemir, Y., Lule, S., Novoa-Carballal, R., Riguera, R., Fernandez-Megia, E., Andrieux, K., Couvreur, P., Capan, Y., Dalkara, T., 2015. Systemically administered brain-targeted nanoparticles transport peptides across the blood-brain barrier and provide neuroprotection. *J. Cereb. Blood Flow Metab.* 35, 469–475. doi:10.1038/jcbfm.2014.220
- Yemisci, M., Gürsoy-Özdemir, Y., Caban, S., Bodur, E., Capan, Y., Dalkara, T., 2012. Transport of a caspase inhibitor across the blood-brain barrier by chitosan nanoparticles. *Meth. Enzymol.* 508, 253–269. doi:10.1016/B978-0-12-391860-4.00013-6
- Yu, Y.J., Atwal, J.K., Zhang, Y., Tong, R.K., Wildsmith, K.R., Tan, C., Bien-Ly, N., Hersom, M., Maloney, J.A., Meilandt, W.J., Bumbaca, D., Gadkar, K., Hoyte, K., Luk, W., Lu, Y., Ernst, J.A., Scarce-Levie,

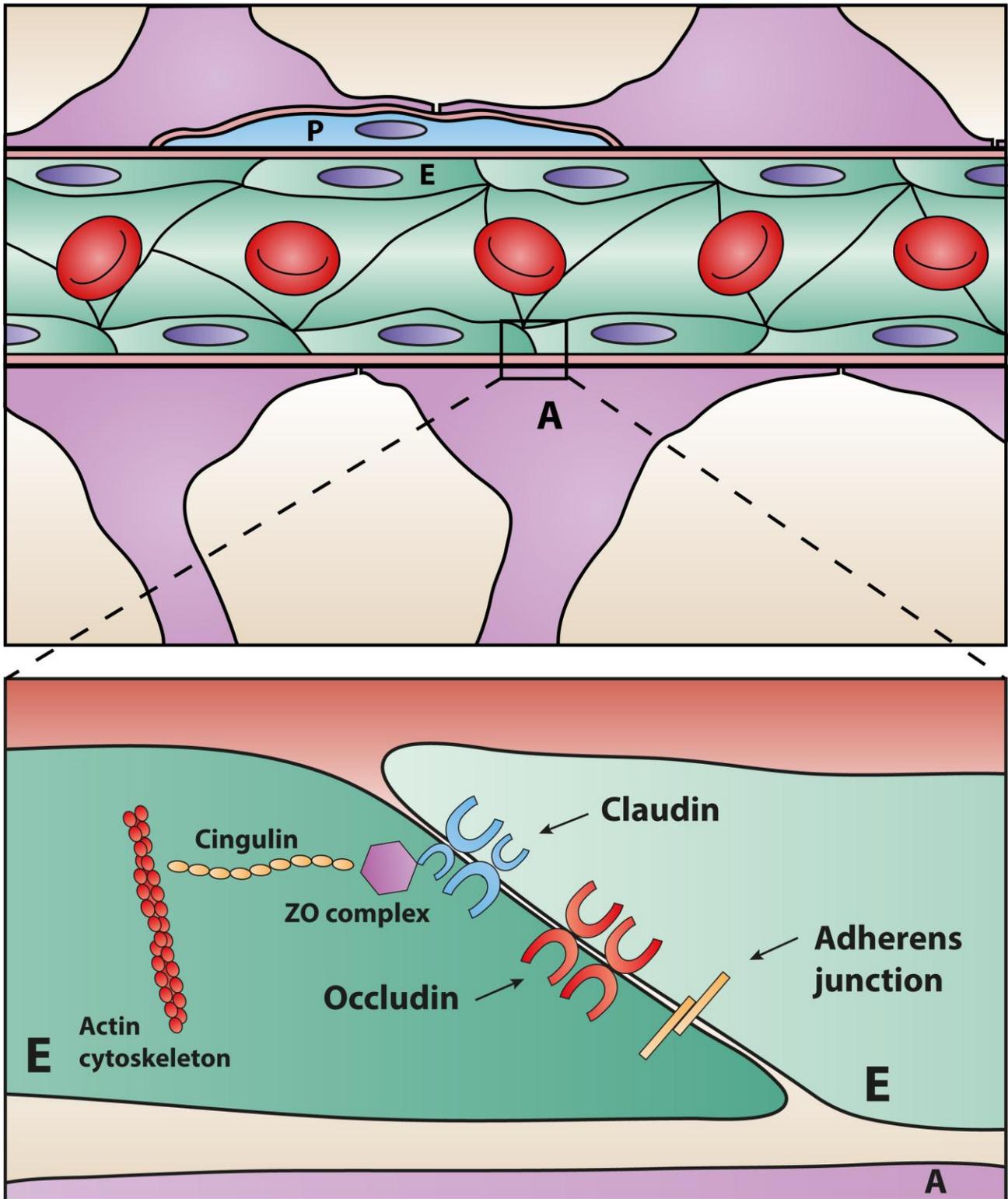
- K., Couch, J.A., Dennis, M.S., Watts, R.J., 2014. Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates. *Sci Transl Med* 6, 261ra154–261ra154. doi:10.1126/scitranslmed.3009835
- Yu, Y.J., Zhang, Y., Kenrick, M., Hoyte, K., Luk, W., Lu, Y., Atwal, J., Elliott, J.M., Prabhu, S., Watts, R.J., Dennis, M.S., 2011. Boosting brain uptake of a therapeutic antibody by reducing its affinity for a transcytosis target. *Sci Transl Med* 3, 84ra44–84ra44. doi:10.1126/scitranslmed.3002230
- Zensi, A., Begley, D., Pontikis, C., Legros, C., Mihoreanu, L., Büchel, C., Kreuter, J., 2010. Human serum albumin nanoparticles modified with apolipoprotein A-I cross the blood-brain barrier and enter the rodent brain. *J Drug Target* 18, 842–848. doi:10.3109/1061186X.2010.513712
- Zensi, A., Begley, D., Pontikis, C., Legros, C., Mihoreanu, L., Wagner, S., Büchel, C., Briesen, von, H., Kreuter, J., 2009. Albumin nanoparticles targeted with Apo E enter the CNS by transcytosis and are delivered to neurones. *J Control Release* 137, 78–86. doi:10.1016/j.jconrel.2009.03.002
- Zhai, M., Wang, Y., Zhang, L., Liang, M., Fu, S., Cui, L., Yang, M., Gong, W., Li, Z., Yu, L., Xie, X., Yang, C., Yang, Y., Gao, C., 2018. Glioma targeting peptide modified apoferritin nanocage. *Drug Deliv* 25, 1013–1024. doi:10.1080/10717544.2018.1464082
- Zhang, C., Mastorakos, P., Sobral, M., Berry, S., Song, E., Nance, E., Eberhart, C.G., Hanes, J., Suk, J.S., 2017a. Strategies to enhance the distribution of nanotherapeutics in the brain. *J Control Release* 267, 232–239. doi:10.1016/j.jconrel.2017.07.028
- Zhang, C., Nance, E.A., Mastorakos, P., Chisholm, J., Berry, S., Eberhart, C., Tyler, B., Brem, H., Suk, J.S., Hanes, J., 2017b. Convection enhanced delivery of cisplatin-loaded brain penetrating nanoparticles cures malignant glioma in rats. *J Control Release* 263, 112–119. doi:10.1016/j.jconrel.2017.03.007
- Zhang, P., Hu, L., Yin, Q., Feng, L., Li, Y., 2012a. Transferrin-modified c[RGDfK]-paclitaxel loaded hybrid micelle for sequential blood-brain barrier penetration and glioma targeting therapy. *Mol. Pharm.* 9, 1590–1598. doi:10.1021/mp200600t
- Zhang, P., Hu, L., Yin, Q., Zhang, Z., Feng, L., Li, Y., 2012b. Transferrin-conjugated polyphosphoester hybrid micelle loading paclitaxel for brain-targeting delivery: synthesis, preparation and in vivo evaluation. *J Control Release* 159, 429–434. doi:10.1016/j.jconrel.2012.01.031
- Zhang, Y., Boado, R.J., Pardridge, W.M., 2003a. In vivo knockdown of gene expression in brain cancer with intravenous RNAi in adult rats. *J Gene Med* 5, 1039–1045. doi:10.1002/jgm.449
- Zhang, Y., Calon, F., Zhu, C., Boado, R.J., Pardridge, W.M., 2003b. Intravenous nonviral gene therapy causes normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental parkinsonism. *Hum. Gene Ther.* 14, 1–12. doi:10.1089/10430340360464660
- Zhang, Y., Pardridge, W.M., 2006. Blood-brain barrier targeting of BDNF improves motor function in rats with middle cerebral artery occlusion. *Brain Research* 1111, 227–229. doi:10.1016/j.brainres.2006.07.005
- Zhang, Y., Pardridge, W.M., 2005. Delivery of beta-galactosidase to mouse brain via the blood-brain barrier transferrin receptor. *J. Pharmacol. Exp. Ther.* 313, 1075–1081. doi:10.1124/jpet.104.082974

- Zhang, Y., Pardridge, W.M., 2001a. Neuroprotection in transient focal brain ischemia after delayed intravenous administration of brain-derived neurotrophic factor conjugated to a blood-brain barrier drug targeting system. *Stroke* 32, 1378–1384.
- Zhang, Y., Pardridge, W.M., 2001b. Conjugation of brain-derived neurotrophic factor to a blood-brain barrier drug targeting system enables neuroprotection in regional brain ischemia following intravenous injection of the neurotrophin. *Brain Research* 889, 49–56.
- Zhang, Y., Schlachetzki, F., Zhang, Y.-F., Boado, R.J., Pardridge, W.M., 2004a. Normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental parkinsonism with intravenous nonviral gene therapy and a brain-specific promoter. *Hum. Gene Ther.* 15, 339–350. doi:10.1089/104303404322959498
- Zhang, Y., Wang, Y., Boado, R.J., Pardridge, W.M., 2008. Lysosomal enzyme replacement of the brain with intravenous non-viral gene transfer. *Pharm. Res.* 25, 400–406. doi:10.1007/s11095-007-9357-6
- Zhang, Y., Zhang, Y.-F., Bryant, J., Charles, A., Boado, R.J., Pardridge, W.M., 2004b. Intravenous RNA interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer. *Clin. Cancer Res.* 10, 3667–3677. doi:10.1158/1078-0432.CCR-03-0740
- Zhang, Y., Zhu, C., Pardridge, W.M., 2002. Antisense gene therapy of brain cancer with an artificial virus gene delivery system. *Mol Ther* 6, 67–72.
- Zhang, Y.-F., Boado, R.J., Pardridge, W.M., 2003. Absence of toxicity of chronic weekly intravenous gene therapy with pegylated immunoliposomes. *Pharm. Res.* 20, 1779–1785.
- Zhao, H., Li, G.-L., Wang, R.-Z., Li, S.-F., Wei, J.-J., Feng, M., Zhao, Y.-J., Ma, W.-B., Yang, Y., Li, Y.-N., Kong, Y.-G., 2010. A comparative study of transfection efficiency between liposomes, immunoliposomes and brain-specific immunoliposomes. *J. Int. Med. Res.* 38, 957–966.
- Zheng, C., Ma, C., Bai, E., Yang, K., Xu, R., 2015. Transferrin and cell-penetrating peptide dual-functioned liposome for targeted drug delivery to glioma. *Int J Clin Exp Med* 8, 1658–1668.
- Zhou, Q.-H., Boado, R.J., Hui, E.K.-W., Lu, J.Z., Pardridge, W.M., 2011a. Brain-penetrating tumor necrosis factor decoy receptor in the mouse. *Drug Metab. Dispos.* 39, 71–76. doi:10.1124/dmd.110.036012
- Zhou, Q.-H., Boado, R.J., Hui, E.K.-W., Lu, J.Z., Pardridge, W.M., 2011b. Chronic dosing of mice with a transferrin receptor monoclonal antibody-glia-derived neurotrophic factor fusion protein. *Drug Metab. Dispos.* 39, 1149–1154. doi:10.1124/dmd.111.038349
- Zhou, Q.-H., Boado, R.J., Lu, J.Z., Hui, E.K.-W., Pardridge, W.M., 2012. Brain-penetrating IgG-iduronate 2-sulfatase fusion protein for the mouse. *Drug Metab. Dispos.* 40, 329–335. doi:10.1124/dmd.111.042903
- Zhou, Q.-H., Boado, R.J., Lu, J.Z., Hui, E.K.-W., Pardridge, W.M., 2010. Monoclonal antibody-glia-derived neurotrophic factor fusion protein penetrates the blood-brain barrier in the mouse. *Drug Metab. Dispos.* 38, 566–572. doi:10.1124/dmd.109.031534

- Zhou, Q.-H., Fu, A., Boado, R.J., Hui, E.K.-W., Lu, J.Z., Pardridge, W.M., 2011c. Receptor-mediated abeta amyloid antibody targeting to Alzheimer's disease mouse brain. *Mol. Pharm.* 8, 280–285. doi:10.1021/mp1003515
- Zhou, Q.-H., Sumbria, R., Hui, E.K.-W., Lu, J.Z., Boado, R.J., Pardridge, W.M., 2011d. Neuroprotection with a brain-penetrating biologic tumor necrosis factor inhibitor. *J. Pharmacol. Exp. Ther.* 339, 618–623. doi:10.1124/jpet.111.185876
- Zhu, C., Zhang, Y., Zhang, Y.-F., Yi Li, J., Boado, R.J., Pardridge, W.M., 2004. Organ-specific expression of the lacZ gene controlled by the opsin promoter after intravenous gene administration in adult mice. *J Gene Med* 6, 906–912. doi:10.1002/jgm.575
- Zlokovic, B.V., 2008. The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 57, 178–201. doi:10.1016/j.neuron.2008.01.003
- Zong, T., Mei, L., Gao, H., Shi, K., Chen, J., Wang, Y., Zhang, Q., Yang, Y., He, Q., 2014. Enhanced Glioma Targeting and Penetration by Dual-Targeting Liposome Co-modified with T7 and TAT. *J Pharm Sci* n/a–n/a. doi:10.1002/jps.24186
- Zuchero, Y.J.Y., Chen, X., Bien-Ly, N., Bumbaca, D., Tong, R.K., Gao, X., Zhang, S., Hoyte, K., Luk, W., Huntley, M.A., Phu, L., Tan, C., Kallop, D., Weimer, R.M., Lu, Y., Kirkpatrick, D.S., Ernst, J.A., Chih, B., Dennis, M.S., Watts, R.J., 2016. Discovery of Novel Blood-Brain Barrier Targets to Enhance Brain Uptake of Therapeutic Antibodies. *Neuron* 89, 70–82. doi:10.1016/j.neuron.2015.11.024

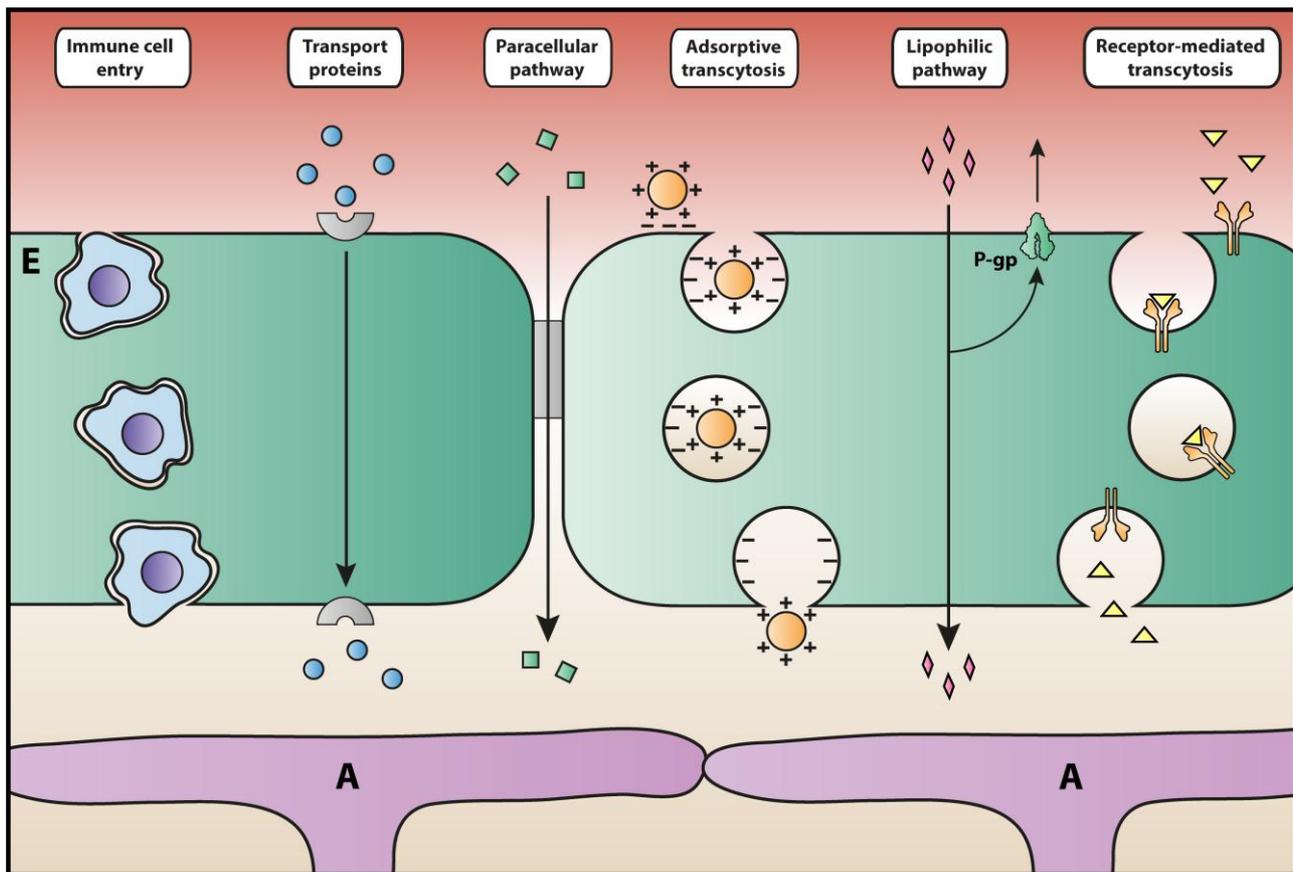
## Figure legends

**Figure 1. The structure and function of the blood-brain barrier.** The blood-brain barrier (top) is composed of brain capillary endothelial cells (E) that line the microvessels of the brain. The structure is supported by pericytes (P) and astrocyte end-feet (A), which are crucial for maintaining the low permeability conditions of the blood-brain barrier. Paracellular transport of molecules is largely restricted due to the tight junction protein complexes (bottom) including claudins and occludin. These tight junctions are further strengthened by the binding to the cell's actin cytoskeleton via adaptor proteins such as cingulin and the zonula occludens (ZO) proteins.



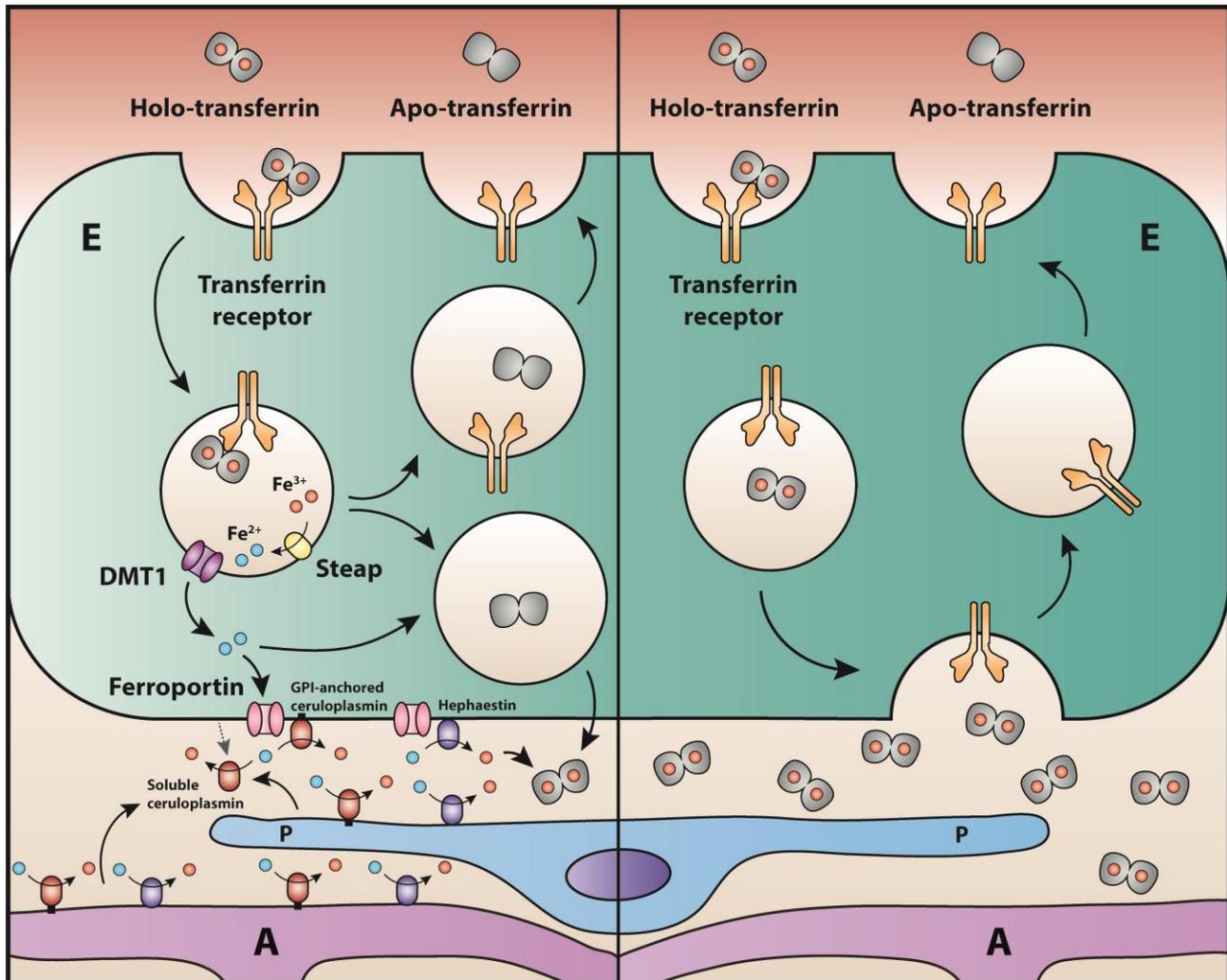
**Figure 2. Transport mechanisms at the blood-brain barrier.** Cells and molecules can enter the brain parenchyma by traversing the blood-brain barrier. As a part of the immune surveillance or during pathological conditions in the brain, immune cells can migrate through the endothelial cell to enter the brain parenchyma and exert their functions. The brain capillary endothelial cells are also characterized by the expression of

several transport proteins that can move nutrients such as sugars or amino acids through the cell. If the molecules are very small and hydrophilic, they may also be able to traverse the endothelial layer at the cell-cell junctions. Furthermore, positively charged molecules can interact with the negatively charged cell surface to initiate a process known as adsorptive transcytosis. Lipophilic molecules can readily enter the intracellular compartment and be transported into the brain parenchyma, but many molecules with this characteristic are also substrates for the P-glycoprotein (P-gp), an important efflux pump. Lastly, plasma proteins can bind to receptors on the endothelial cell surface to initiate a process, whereby the receptor and bound molecules are transported through the cell. This is known as receptor-mediated transcytosis.



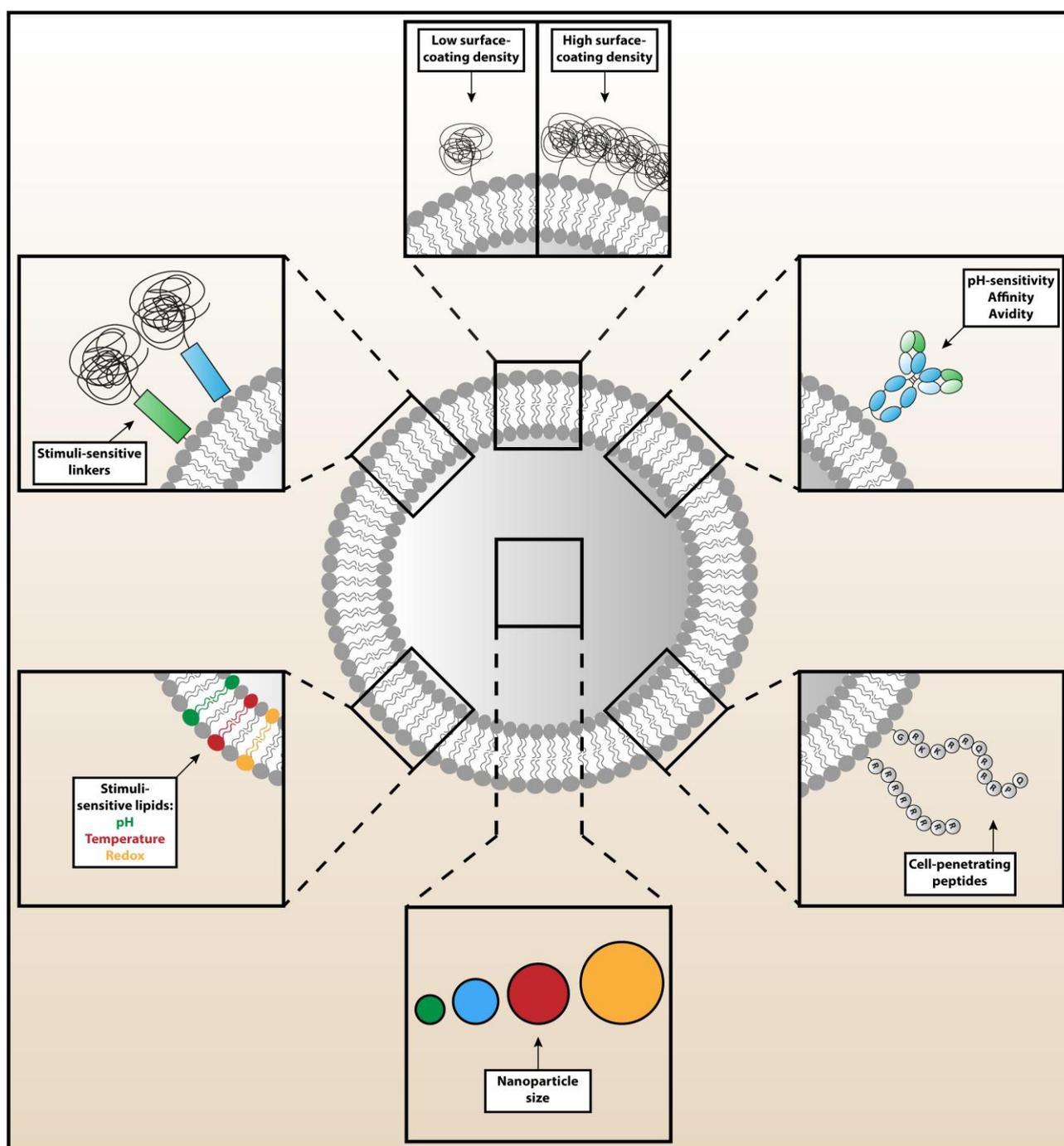
**Figure 3. Iron uptake across the blood-brain barrier.** Brain iron uptake is thought to occur via one of two currently discussed models. The first model (left) involves endocytosis of the Tf-TfR complex followed by acidification of the resulting endosome. The reduction in pH during the acidification reduces the affinity of the two iron atoms for the Tf protein, and thus, they dissociate. The ferric iron is then reduced by ferrireductases present in the endosome membrane (e.g. Steap proteins) to yield ferrous iron, which can be transported out of the endosome by DMT1. The ferrous iron can subsequently associate with the iron exporter, ferroportin, which can pump iron into the brain parenchyma. This process is, however, dependent on the activity of a ferroxidase (e.g. hephaestin or ceruloplasmin). These ferroxidases can be found both soluble and associated with the plasma membrane of the brain capillary endothelial cells (E), the pericytes (P), and the astrocytic end-feet (A). Release of protein-bound iron has also been proposed in this model, but depends on an intracellular pool of Tf

molecules in the BCECs rather than a transcytosis process of Tf molecules from the systemic circulation. The second model (right) also involves endocytosis of the Tf-TfR complex, but instead of having the iron atoms releasing during the endosomal acidification, the TfR itself transcytoses across the brain capillary endothelial cell to present the holo-Tf protein to the brain parenchyma. Subsequently, the Tf protein will be released into the brain parenchyma, although dissociation of the iron atoms at the abluminal membrane has been suggested.



**Figure 4. Modifications of nanoparticles to improve brain uptake.** The efficiency of TfR-targeted nanomedicines may be improved by exploiting the impact of adding new features to the drug delivery system (here the liposome). First, varying the surface coating density may be of great importance, since this can affect the nanoparticle's ability to move in the brain parenchyma after transcytosis. To obtain transcytosis, its targeting ligand should also be endowed with the ability of dissociating the TfR during the intracellular sorting process, e.g. by modulating the pH-sensitivity, affinity or avidity. The transendothelial transport may also be enhanced by dual-targeting the nanoparticle with a cell-penetrating peptide. The size of the nanoparticle is also an important aspect to consider since different sizes can initiate different kinds of intracellular sorting, where the one for smaller nanoparticles may be more favorable for transport across the blood-brain barrier. If the

nanoparticle is not expected to leave the endothelial cell after endocytosis, or if the nanoparticle is stuck in the basement membrane after transcytosis, designing the nanoparticle to become activated in either of these compartments could also be a favorable approach. One way is to incorporate stimuli-sensitive lipids into liposomes that make the liposome disintegrate in specific microenvironmental conditions. The same microenvironments could also be used as an activator for cleaving stimuli-sensitive linkers spacing the surface coating and the nanoparticle.



**Figure 5. Modifications of antibody-based medicines to improve brain uptake.** Several strategies exist to improve the brain uptake of antibodies with a therapeutic efficacy within the brain. The most popular strategy

is to transport with the TfR across the blood-brain barrier with antibodies or antibody fragments that bind the TfR with high affinity. Conversely, reducing the affinity of the antibody may induce a more favorable route of intracellular sorting, wherein the anti-TfR antibody avoids being targeted to the lysosomes for degradation. This strategy also maintains the expression of TfRs at the BBB, which can be reduced when targeting with high affinity antibodies. Many of the beneficial effects of targeting the TfR with low affinity antibodies can also be obtained using a monovalent strategy. This strategy ensures that the anti-TfR antibodies cannot engage several TfRs simultaneously, which is known to be detrimental to further transport. Monovalency can be incorporated into the design of the therapeutic antibody either in 1) a classical bi-specific antibody format, where the TfR-targeting is placed in one of the variable domains, 2) a brain shuttle format, where a single chain antibody fragment is engineered into the therapeutic antibody via a linker, or 3) a “transport vehicle” format, where the TfR-binding epitope is incorporated into the Fc domain of the therapeutic antibody. The latter two strategies also allow for the therapeutic target to be engaged in a bivalent format. Lastly, the binding between the transporting antibody and the TfR can be sensitive to changes in pH, which will detach the antibody when the endosomal acidification takes place during TfR endocytosis and intracellular sorting.

