Transcytosis and the Neurophysiological Complications Involved in the Delivery of Drugs Across the Blood-Brain Barrier in Alzheimer's Disease

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SUMMARY

The medical field relies heavily on the collection and development of knowledge required to design novel drugs, as well as formulate innovative and effective methods of drug delivery, particularly with reference to neurophysiological diseases. Alzheimer's is one of many chronic neurodegenerative diseases and impacts more than 40 million people worldwide. Diseases such as Alzheimer's can be devastating for a patient and those caring for them, and when coupled with other ailments common to aging populations they can significantly decrease quality of life. While some treatments currently exist for Alzheimer's disease, they serve only as short-term symptom relief by causing an increase of acetylcholine in the brain. Extensive research is currently underway to identify and design new drugs capable of sustaining long-term slowing of, or arrest Alzheimer's disease progression. One of the main challenges to successfully delivering such a drug, however, is traversing the blood-brain barrier. Therefore, the aim of this literature review is to examine two methodologies of drug delivery through the blood-brain barrier currently undergoing development: adsorptive- and receptor-mediated transcytosis. These methods focus on the interactions of the molecular carrier and the blood brain barrier and its chemical and physiological characteristics to assist in drug delivery. This review also investigates the stage of development that each of these delivery techniques are currently in, assesses the potential for the delivery method to be used in the active treatment of neurological diseases, and evaluates the benefits and disadvantages of each method.

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that currently affects more than 40 million people worldwide, and this number is expected to increase exponentially in the coming decades (Esquerda-Canals et al., 2017). AD is characterized primarily by cognitive impairment and neurodegeneration - the result of synaptic damage and subsequent neuronal loss. At a molecular level, this results from the formation of amyloid-β-containing plaques and neurofibrillary tangles composed of hyperphosphorylated tau protein (Crews and Masliah). These features then manifest themselves on a spectrum from early

memory changes to functional dependence, and eventually death (Neugroschl and Wang, 2011).

Currently, AD is treated symptomatically, however such treatments exhibit limited success since they can only counter surface-level neurotransmitter imbalances (Yiannapoulou and Papageorgiou, 2013). There have recently been significant strides made in the research of disease-modifying drugs which attempt to treat AD at its source. Unfortunately, these new drugs must overcome one main obstacle - traversing or circumventing the blood-brain barrier (BBB) to effectively reach the target areas of the brain.

BLOOD-BRAIN BARRIER

The brain is a highly important organ that, despite its dense vasculature, is extremely sensitive to circulating compounds (Herve, Ghinea, and Scherrmann, 2008). Thus, there needs to be constant and strict control over which substances are allowed to come into direct contact with the brain. The BBB is known to perform this function.

The BBB is defined by most researchers as a non-fenestrated microvascular endothelium with incredibly narrow tight junctions, few alternate transport pathways, and elevated levels of degrading enzymes (Herve, Ghinea, and Scherrmann, 2008; Rocha, 2013). This barrier serves to maintain homeostatic stability in the environment created by the brain parenchyma through the control of blood vessels and selective transport systems (Betsholtz, 2014). Almost all water-soluble compounds are prevented from entering the brain via the usual paracellular pathway - through aqueous channels - and this, unfortunately, includes peptide drugs among many others. Hence, the BBB is one of the main obstacles for the successful delivery of drugs to the central nervous system (CNS), which is essential for the treatment of neurodegenerative diseases such as AD (Banks, 2012). Not all the mechanisms that facilitate the function of the BBB are fully understood, and therefore it is a greater challenge for researchers to circumvent the mechanisms and processes which stop most drugs from passing through it (Betsholtz, 2014). As such, this literature review focuses on existing and better

Table 1: A list of search terms used to search for literature used in this review, categorized by section in review.

General Background	Absorptive-mediated Transcytosis	Receptor-mediated Transcytosis
Blood-brain barrier	Negative blood-brain barrier	Receptor-mediated transcytosis
Blood brain barrier	Absorptive mechanisms	Receptor ligand systems
Alzheimer's disease	Absorptive-mediated transcytosis	Transferrin Receptor Antibody
Alzheimer	Adsorptive-mediated transcytosis	Transferrin receptor
Structure	Putrescine cationization	Targeting Receptor-Mediated Transport
Function	Cationized albumin	Transferrin-mediated transcytosis
Acetylcholinesterase inhibitor	Cell-penetrating peptide	
Delivery system Alzheimer's disease		
Drug delivery CNS		

understood transport systems capable of being harnessed as novel and effective drug delivery methods to treat neurological diseases such as AD, namely methods of transcytosis.

TRANSCYTOSIS

The BBB maintains cerebral homeostasis by admitting or preventing particular substances in the bloodstream from entering the brain (Weiss et al., 2009). The transport of nutrients, such as amino acids, glucose, or other small molecules through brain microvascular endothelial cells (BMECs) is done through the process of transcytosis - the vesicular transport of macromolecules from one side of a cell to the other (Lajoie and Shusta, 2015; Tuma and Hubbard, 2003). This is done in order to enable and ensure proper neuronal and supporting cell function and growth. The BBB's selectivity is based on the existence of specific surface receptors that are expressed on BMECs in order to bind and signal the endocytosis of ligands carrying desired molecules or minerals (Yu, Li, Tao and Wang, 2015). It is evident based on recent literature that the advancement in our understanding of endogenous transport at the BBB will support and cultivate the development of successful procedures and nanoparticle-based strategies for transporting and delivering biologics capable of treating neurological diseases to the brain. These particles may be between 1 and 100 nanometres in diameter.

ABSORPTIVE-MEDIATED TRANSCYTOSIS

There are multiple forms of transcytosis that occur across the BBB to transport certain blood plasma constituents to the parenchyma. One of the most fundamental types is absorptive-mediated transcytosis (AMT), which have been shown to have the potential to facilitate successful biologic delivery (Herve, Ghinea and Scherrmann, 2008). AMT involves the endocytosis of a cationic molecule at the luminal surface of the BBB through interactions with anionic particles on the plasma membrane, followed by exocytosis from the abluminal surface (Herve, Ghinea and Scherrmann, 2008). Moieties expressed on the luminal surface of BMECs are suited for interactions with ligands, that are ideally polycationic, which leads to binding, membrane invagination, and their eventual uptake (Herve, Ghinea, and Scherrmann, 2008). The BBB is already suited for AMT due to the many membrane surface regions of the luminal face of BMECs that are negatively charged at physiological pH (7.4) (Herve, Ghinea, and Scherrmann, 2008). Negative charges stem from the sialo-glycoconjugates and heparan sulfate proteoglycans which make up the glycocalyx of the BBB's luminal surface (Herve, Ghinea, and Scherrmann, 2008; cited as Vorbrodt, 1989). In addition, since this is an active transport process which requires metabolic energy in the form of adenosine triphosphate, the BBB is an ideal location, having five times more mitochondria than any other peripheral endothelium (Herve, Ghinea, and Scherrmann, 2008).

RECEPTOR-MEDIATED TRANSCYTOSIS

Another transport mechanism of interest is receptor-mediated transcytosis (RMT) (Preusch, 2007). RMT is the receptor-mediated uptake of a ligand on one side of the cell, vesicular transport across the cell, and exocytosis of vesicle contents on the opposite side, much like AMT. A consequence of RMTs molecular process and function of acting as a key transport system at the BBB is the availability of this system for 'hijacking' to facilitate cerebral biologic delivery (Rip et al., 2010). RMT makes use of so-called 'trafficking machinery' on and within BMECs to deliver a range of proteins including transferrin (Tf), insulin, leptin, and lipoproteins to the brain (Dehouck et al., 1997; Descamps et al. 1996; Duffy and Pardridge, 1987; Golden, Maccagnan and Pardridge, 1997; as cited in Lajoie and Shusta, 2015). The RMT process involves four steps (Figure 1). Initially, a circulating ligand binds to a cognate transmembrane receptor expressed on the luminal (blood side) plasma membrane. Endocytosis then occurs through membrane invagination and the formation of an intracellular vesicle that contains the receptor-ligand complex (Parkar et al., 2009, as cited in Lajoie and Shusta, 2015). The newly formed intracellular vesicle can be directed to various destinations across the endothelial cytoplasm through the use of the cell's vesicular and endolysosomal trafficking machinery (Brooks, 2009; Rodriguez-Boulan, Kreitzer and Müsch, 2005, as cited in Lajoie and Shusta, 2015; Sharma et al. 2016). In the case of transcytosis, the vesicle is shuttled to the basolateral (brain-side) membrane where exocytosis occurs, releasing the vesicular contents into the parenchyma (Strazielle

and Ghersi-Egea, 2013, as cited in Lajoie and Shusta, 2015).

ABSORPTIVE MEDIATED TRANSCYTOSIS VIA CATIONIZATION OF PEPTIDE DRUGS

The initiation of AMT from cationic particles binding to anionic particles in the plasma membrane can be harnessed by drug delivery systems. There are several possible ways to cationize a peptide drug to enhance theraputic delivery, a number of which involve carbodiimide-mediated amidation of carboxylic acid groups to directly deliver the peptide without a cationic import carrier (Herve, Ghinea, and Scherrmann, 2008).

CATIONIZATION OF AMYLOID-BETA ANTIBODY

Cationic molecules have displayed improved uptake to the brain in several experiments using mice. For example, cationized bovine serum albumin-PEG-PLA demonstrated a significant increase in transport to the brain, compared to non-cationized albumin-PEG-PLA (Lu et al., 2005). In a study related to AD, Syvänen, Edén, and Sehlin (2017) investigated the effect of cationization of an antibody fragment with the polyamine putrescine as a means to increase the surface charge of the antibody and potentially improve its interaction with the luminal face of endothelial cells to penetrate the BBB. The researchers used an amyloid-beta protofibril selective antibody h158, which was cleaved enzymatically to a F(ab')2 fragment then cationized by cross-linking it to the polyamine putrescine using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The percentage of fragments that reached the brain was determined using radiolabeling with iodine-125. After comparing the unmodified antibody fragment to the cationized fragment in the brains of the mice, it was demonstrated that cationization led to lower concentrations of the antibody remaining in the blood, whereas the concentrations in the brain were at the very least similar, and at times increased (Syvänen, Edén, and

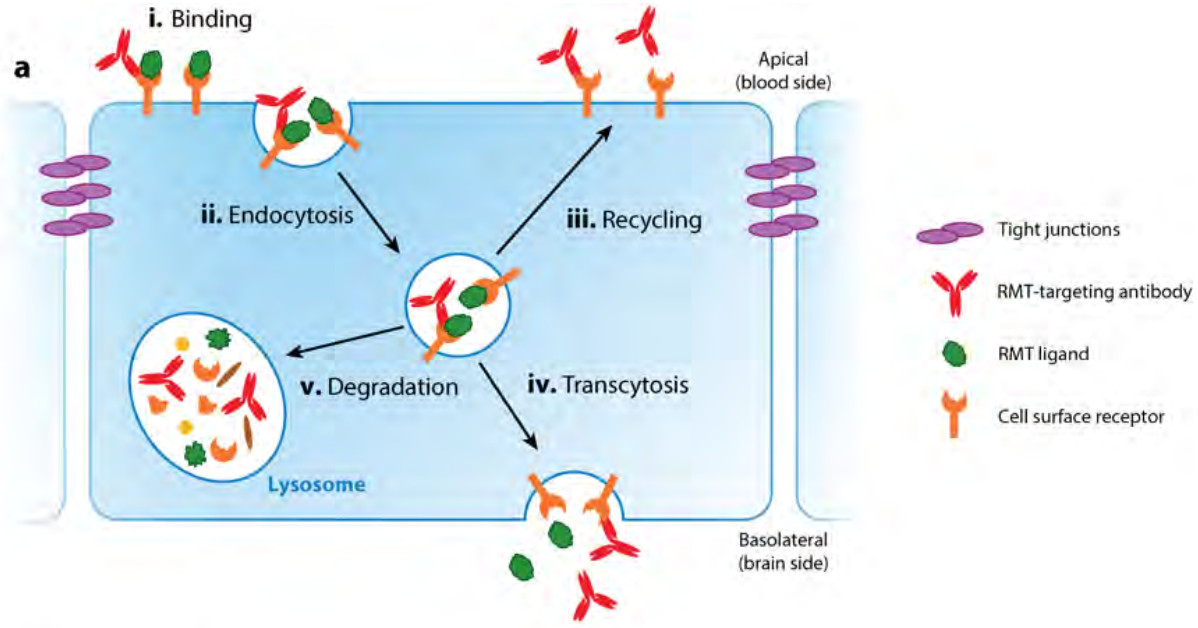

Figure 1: An illustration of RMT in a BMEC. (i) Initially, a ligand binds to a cognate transmembrane receptor on the luminal plasma membrane. (ii) Endocytosis occurs through membrane invagination, which generates receptor-ligand containing intracellular vesicles. (iii) The intracellular vesicle can be directed to various destinations across the endothelial cytoplasm via the cell's vesicular and endolysosomal trafficking machinery. (iv) During transcytosis, the vesicle is shuttled to the basolateral membrane where exocytosis occurs. (v) Vesicles can also be sent to a lysosome to be degraded (Lajoie and Shusta, 2015).

Sehlin, 2017). Moreover, the cationized fragments were ostensibly present in the third ventricle and front cortical region of mice brains using single photon emission computed tomography imaging (SPECT) (Figure 2C), which were known to contain large amounts of amyloid-beta plaques (Syvänen, Edén, and Sehlin, 2017). The cationized fragments did not visualize beta-amyloid plaques (Figure 2B and 2C), but were hypothesized to represent the soluble amyloid-beta species around them. In addition, enzyme-linked immunosorbent assays allowed researchers to determine that cationization of the F(ab')2 antibody fragment did not alter its binding affinity to amyloid-beta protofibrils (Syvänen, Edén, and Sehlin, 2017). Therefore, cationized antibody fragments, and perhaps other cationized peptide drugs, present a viable option for improved therapeutic uptake. Another experiment conducted in by Agyare et al. (2008) supported the claim that putrescine-cationization can transport several types of particles through the BBB, as chitosan nanoparticles coated with putrescine modified antibodies were able to be transported successfully to the brain.

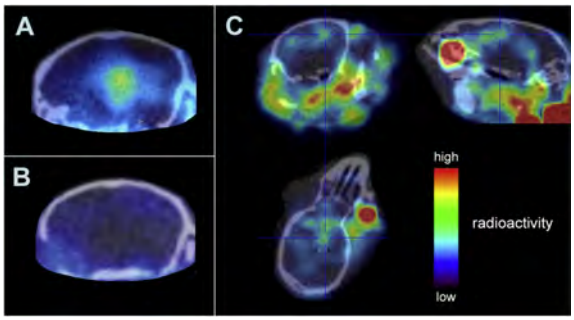

Figure 2: The coronal view of SPECT images obtained of the third ventricle in the brain of 17–18 month old tg-ArcSwe mice two hours after injection with either (A) [125I]pF(ab')2-h158 or (B) [125I]F(ab')2-h158. (C) Coronal, sagittal and transverse images obtained from the frontal cortical area of the mouse injected with [125I]pF(ab')2-h158. The blue cross-hair indicates the same position in all three images. A legend assists in qualifying the level of radioactivity represented by the image, proportional to accumulation of antibodies, which demonstrate increased uptake of the antibody fragment when cationized (Syvänen, Edén, and Sehlin, 2017).

ABSORPTIVE-MEDIATED TRANSCYTOSIS WITH CELL-PENETRATING PEPTIDES

If the drug itself is not directly cationized post-development, researchers may find cell-penetrating-peptides (CPPs) as appropriate choices to aid transport into the brain by AMT. CPPs are short peptides, less than thirty amino acids long, with a net positive charge and amphipathic characteristics that are capable of entering living cells without inducing cytolysis (Chen and Liu, 2012). These peptides successfully interact with lipid membranes by adopting a certain secondary structure upon contact. In fact, CPPs increase doxorubicin transport into rat brains thirty-fold (Chen and Liu, 2012). In an in vivo experiment with a murine brain microvascular endothelial cell model, CPP-modified liposomes (Figure 3) significantly increased the transport ratio of rivastigmine solution to the CNS (Yang et al, 2013). Rivastigmine is an FDA- and Health Canada-approved acetylcholinesterase inhibitor drug currently used to treat Alzheimer's disease. The study also demonstrated no significant difference between parameters such as nasal mucosa morphology and cilia movement in rivastigmine CPP-liposome formulations and normal saline, indicating that the drug system did not cause notable toxicity (Yang et al, 2013). The most promising type of CPP in this study was found to be L-penetratin, though there are other popular peptides such as TAT and the Syn-B vector as well. Similar to cationization, several studies cite the success of CPPs to enhance the systemic delivery of other particles through the blood-brain barrier, namely polymeric nanoparticles (Patel et al., 2012).

TARGETING BIOLOGICS AT THE BRAIN VIA RECEPTOR-MEDIATED TRANSCYTOSIS

The method of manipulating receptor-mediated pathways to allow for the delivery of biologics to the brain involves the conjugation of a receptor-targeting signal molecule with a therapeutic of interest (Broadwell et al., 1996; Friden et al., 1991; Pardridge, Buciak, and Friden, 1996, as cited in Lajoie and Shusta, 2015). The use of RMT has

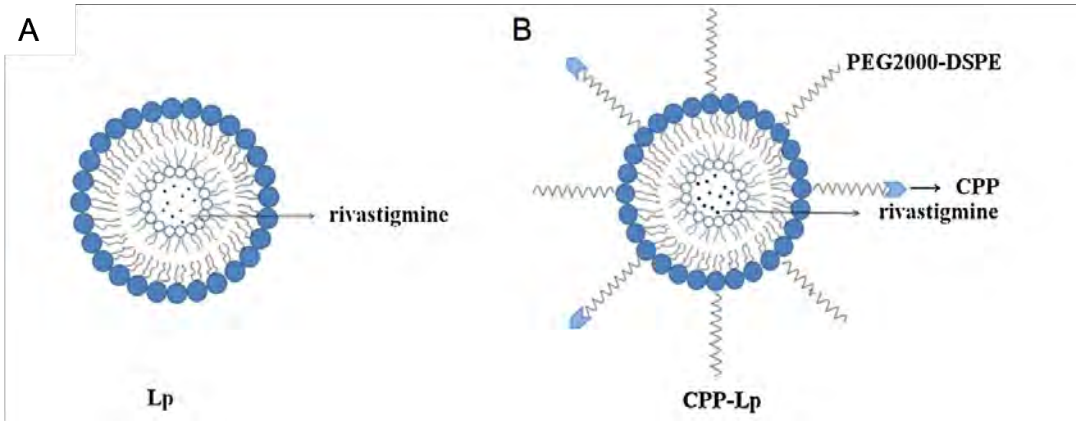

Figure 3: A Schematic diagram of (A) liposomes and (B) CPP-modified rivastigmine liposomes (CPP-Lp) carrying a rivastigmine displaying membrane bilayer and other attached constituents (Yang et al., 2013).

been developed to allow for the delivery of many different biologics, including monoclonal antibodies (mAbs), recombinant proteins, RNA, DNA, and nanomedicines (Lajoie and Shusta, 2015). There are generally two methods of coupling the biologic to the RMT-targeting moiety, the first of which involves a direct chemical linkage or molecular fusion, and the second the generation and use of loaded nanoparticles such as liposomes (Jones and Shusta, 2007, as cited in Lajoie and Shusta, 2015).

TRANSFERRIN

One of the first RMT system proteins to be studied and exploited for neurological drug delivery was the transferrin receptor (TfR). Binding experiments have shown that the TfR, a glycoprotein that is approximately 80-kDa in length, is highly expressed on BMECs of both rats and humans, and that the transport of iron to the brain in both species involves the RMT of Tf (Cabezón et al, 2015; Descamps et al. 1996; Fishman et al., 1987; Lajoie and Shusta, 2015; Zuchero et al., 2016). In iron transport, after the binding of diferric Tf to the TfR, the receptor-ligand complex is endocytosed into the cell where a lysosome fuses with and causes dissociation of the iron, which can subsequently be transferred elsewhere in the cell for use or storage (Descamps et al. 1996). There still exists a dispute over the level of Tf recycling by the BMECs and the rate at which Tf is transcytosed (Clark and Davis, 2015; Descamps et al. 1996; Wiley et al., 2013; Yu et al., 2011). Recent studies have explored new variations in the manipulation of this trafficking machinery, which have encouraged new and ongoing investigations involving the RMT pathway.

TRANSFERRIN VECTORS

Many variations of nanoparticles, as well as therapeutic drug molecules, can be conjugated to either Tf proteins or TfR-targeted monoclonal antibodies. The TfR antibody (anti-TfR) has been shown to bind to a different site compared to a Tf protein, and as such unlikely to interfere with the endogenous Tf circulating in the blood (Sharma et al. 2016). Therefore, several recent studies have presented, and in many cases developed, improvements to the application of monoclonal antibodies and nanoparticles for targeting the RMT of Tf (Bien-Ly et al., 2014; Clark and Davis, 2015; Wiley et al., 2013; Yu et al. 2011).

Although antibodies and antibody-conjugated vectors have been shown to enter, and sometimes fully transcytose across BMECs, there have long been reported caveats to the methodology of RMT manipulation and the use of Tf (Gosk et al. 2004; Lajoie and Shusta, 2015). The primary issue that has perpetuated within the study and manipulation of Tf-RMT, with respect to the delivery of nanoparticles and biologics across the BBB, has been the transfection levels of the Tf-conjugated delivery vectors (Gosk et al. 2004; Lajoie and Shusta, 2015). The high concentration of Tf protein in circulation competes with the transferrin on the nanoparticle system (Girão da Cruz, Simões and Pedroso de Lima, 2004, as cited in Sharma et al. 2016). Fortunately, there have been two different approaches to rectify this issue.

There are other factors to be considered when evaluating the appropriateness of utilizing RMT as a delivery strategy, and these will be discussed later in this review.

MODIFYING MONOCLONAL ANTIBODY AFFINITY

Numerous studies have been conducted to examine the potential of anti-TfR antibodies to target the TfR and have presented findings revealing the potential for their endocytosis into BMECs. However, whether or not this leads to antibody release and therapeutic accumulation in brain parenchyma has remained controversial. Following this, data has shown high levels of anti-TfR degradation by lysosomes, leading to the break down and expulsion of the antibody, and thus any attached biologic (Lajoie and Shusta, 2015). In order to act as an effective targeting system, an anti-TfR antibody must be able to deliver attached therapeutics to the brain parenchyma at doses high enough to cause a measurable therapeutic effect.

Traditionally, monoclonal antibody affinities for the Tf receptor were designed to be extremely high in experiments with Tf-RMT and anti-TfR antibodies. The use of high-affinity anti-TfR antibodies ensures specific binding to the TfR, which leads to uptake of the antibodies to BMECs even at low blood concentrations. Their high affinity for TfRs, however, has been found to likely reduce the probability of the antibody being released from the cerebral vasculature or TfR-carrying-endosome, and thus potentially prevents accumulation of antibodies in the parenchyma (Figure 4). A study by Yu et. al (2011) was conducted to determine the implications of lowering anti-TfR antibodies' affinity on antibody EC uptake, transcytosis, and potential for these antibodies to function as a successful therapeutic mechanism. Trials involved the injection of either a trace or therapeutic dose into mice intravenously, and subsequent brain uptake was measured after 1 and 24 hours. It was found initially that while high-affinity anti-TfR antibodies could accumulate in the brain at both trace and therapeutic levels, they remained predominantly in the cerebral vasculature and did not enter the parenchyma (Figure 4A and B). These observations indicate that only minimal transcytosis occurred. Researchers then explored whether lowering the antibody's affinity for TfRs would allow for greater accumulation in the parenchyma, which was done by introducing

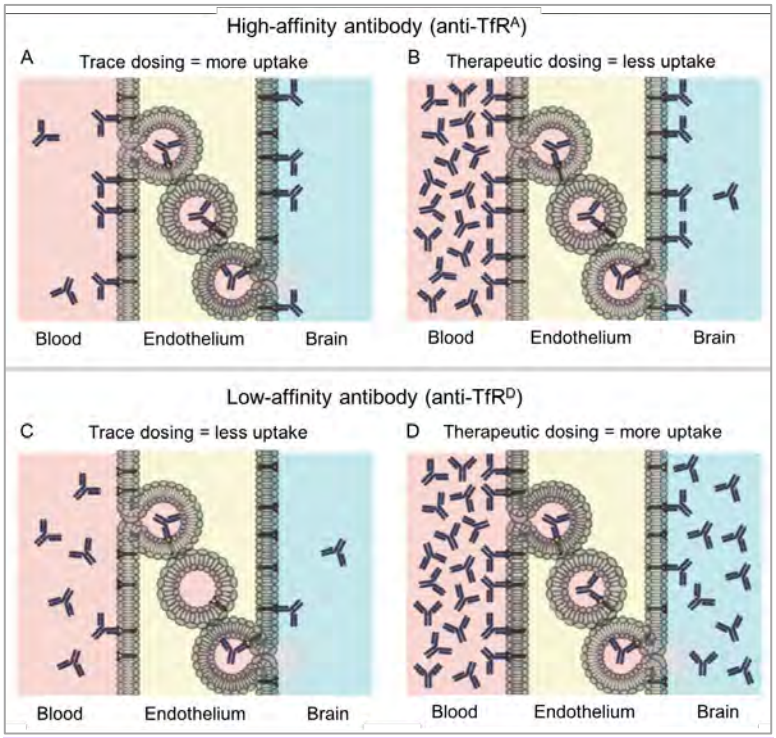

Figure 4: A Model representing the inverse relationship between an antibody's Affinity for TfR and its RMT to the brain. When a trace dose is administered, higher-affinity antibodies (A) will bind more readily to receptors expressed on the luminal side of BMECs compared to the trace dose of lower-affinity antibodies (C), meaning more high-affinity antibodies are available to enter the brain parenchyma. When a therapeutic dose is administered, the saturation of BMECs will result in antibody binding to receptors on the luminal side of the BBB epithelium regardless of antibody affinity. The dissociation of lower-affinity antibodies (D), however, will be more likely, and result in the higher accumulation of antibodies in the brain parenchyma compared to the higher-affinity antibodies (B) (Yu et al., 2011).

alanine mutations into the complementarity-determining regions of anti-TfR antibodies. This generated antibody variants with differing affinities for the TfR, and these variants were then evaluated for uptake after trace and therapeutic intravenous dose administrations. It was found that when delivered in trace doses, anti-TfR antibodies demonstrated a direct correlation between affinity and brain uptake, with lower-affinity antibodies showing the lowest uptake (Figure 4C). Conversely, when therapeutic doses were administered, there was an inverse correlation between affinity and brain uptake (Figure 4D). This represents a higher level of successful transcytosis of lower-affinity antibodies. The antibody with the lowest affinity in the experiment was even found to have a more than a fivefold increase in brain antibody concentration compared to control Immunoglobulin G (IgG) 24 hours after injection.

When comparing the high- and low-affinity antibodies at low blood concentrations, Yu et al. (2011) found that the high TfR affinity antibodies bound tightly to brain endothelial receptors, while low-affinity antibodies were less likely to bind and remain bound to the luminal side of the cerebral vasculature. This led to the conclusion that when BMECs are not saturated with low-affinity antibodies, their RMT to the brain is reduced. At higher doses, however, the luminal TfR would be saturated regardless of antibody affinity, and so there would be similar endothelial uptake for both types of antibodies. In this case, low-affinity antibodies would demonstrate a higher level of brain accumulation through an increased dissociation from TfR and release into the brain. Additionally, it was found that having a lower affinity antibody decreases the probability of receptor-mediated efflux out of the parenchyma, since concentrations in the brain are likely to no longer be saturating. In order to further address the parenchymal distribution of antibodies at therapeutic doses, Yu et. al (2011) compared antibody distribution in the brain for each affinity variant. The results of brain section visualizations from stained fluorescent anti-human secondary IgG were that the high-affinity anti-TfR antibody localized predominantly in the vasculature, but in contrast, lower-affinity variants localized with the neuronal marker neuronal nuclei, suggesting their broad distribution in the brain parenchyma surrounding neurons. This supplemented the observation that the low-affinity antibodies possessed a greater propensity to be transcytosed and distributed through the brain than high-affinity antibodies. These findings have ultimately demonstrated how the manipulation of antibody affinity can be used to increase their RMT and parenchyma delivery, which may present a partial solution to the issue of delivering therapeutics in such a manner.

MODIFYING NANOPARTICLE AVIDITY

The branch of RMT study that analyses the potential therapeutic use of antibodies and antibody-bound biologics is one of many. To the same extent, the investigation into the influence of antibody affinity on transcytosis is just a single focus of research and development with the goal of adapting RMT as a successful therapeutic method for treating neurological diseases. An alternative approach taken to develop a successful method of delivering therapeutic agents across the BBB was examined by Wiley et al. (2013), and Clark and Davis (2015). These studies examined the avidity, or overall stability between antibodies and antigens, of Tf-containing nanoparticles. Avidity is governed by the intrinsic affinity of the antibody for the epitope - the valency of the antibody and antigen - and the geometric arrangement of the interacting components.

One of the main goals of research into Tf-RMT is to find or design a nanoparticle with the ability to cross the BBB after a therapeutic dose and deposit a biologic at a concentration high enough to be capable of treating neurological diseases (Yu et al., 2011). Wiley et al. (2013) examined the capability of Tf-containing gold nanoparticles (AuNPs) to cross the BBB. This study found that Tf-containing 80-nm gold AuNPs with near-neutral zeta potentials were capable of entering the parenchyma after transcytosis when their avidity to TfRs was low, whereas high-avidity AuNPs remained strongly associated with the BMECs of the BBB.

Building on the findings from Wiley et al. (2013), Clark and Davis (2015) sought to further develop the avidity-tuned AuNP design and increase the ability of such Tf-containing nanoparticles to reach the parenchyma. To do this, they generated chemical linkages between the nanoparticle cores and Tf that were capable of cleaving at moderately acidic pH levels (Figure 5). The design of these nanoparticles provided them with high-avidity interactions with TfRs on the luminal face of the BBB, as well as the ability to undergo cleavage of the linkage during transcytosis (Clark and Davis,

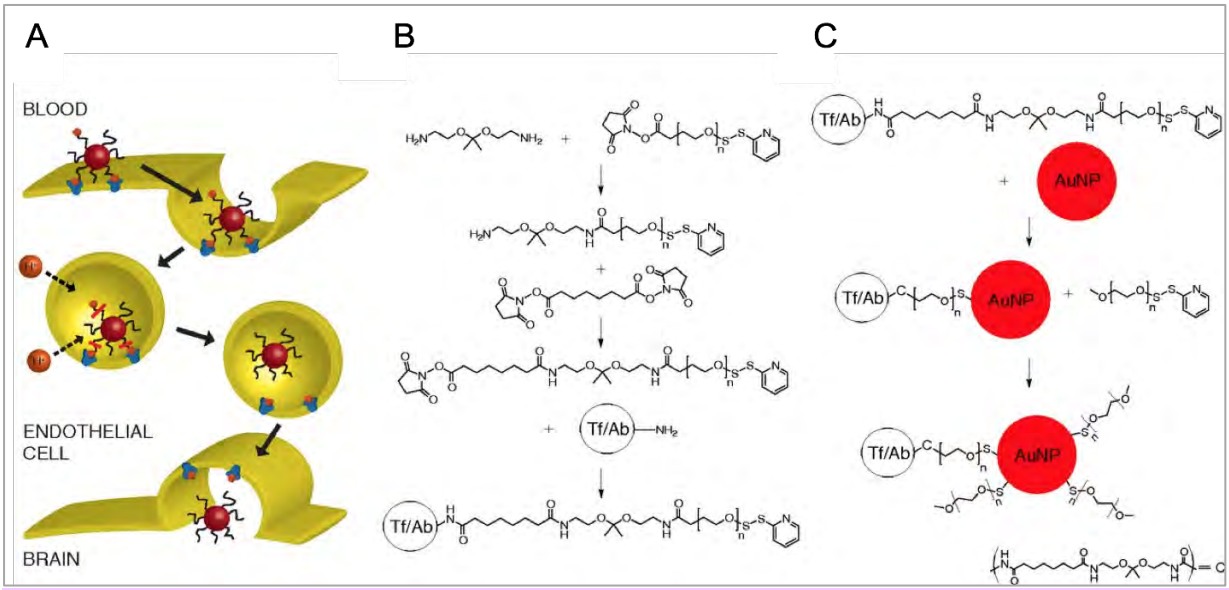

Figure 5: (A) The proposed mechanism of transcytosis for Tf-containing nanoparticles with acid-cleavable linkages. After endocytosis, the acidification of the endosome causes the separation of Tf ligand from the nanoparticle core, which allows the movement of the nanoparticle to the parenchyma to complete transcytosis. (B) The preparation of acid-cleavable DSS-DAK-PEG-OPSS and the addition to a targeting ligand (the anti-TfR antibody) to create the cleavable conjugate. (C) The addition of the anti-TfR antibody-DAK-PEG-OPSS ligand followed by excess mPEG-SH to prepare targeted gold nanoparticles (Clark and Davis, 2015).

2015; Mellman, Fuchs and Helenius, 1986, as cited in Clark and Davis, 2015; Sade et al., 2014). This inducible cleavage meant that the nanoparticles could become unbound during the process of vesicle endocytosis into, and transport through BMECs, and would subsequently be readily available for release to the parenchyma. After testing using an in vitro model, the targeted acid-cleavable AuNPs demonstrated an increased ability to cross the BBB, and in vivo they were able to enter the parenchyma of mice in far greater amounts after systemic administration than similar high-avidity nanoparticles containing non-cleavable Tf.

DEGRADATION

To further improve their application, Yu et al. (2011) went on to design bispecific antibodies capable of binding with low affinity to TfR, and with high affinity to the enzyme β-secretase (BACE1). BACE1 converts amyloid precursor protein into amyloid-β peptides, including those associated with Alzheimer's disease (Roberds, 2001; Vassar, 1999 as cited in Yu et al., 2011). Monospecific antibodies to BACE1 (anti-BACE1) were also tested in this investigation as a means to quantify changes in binding and RMT of the bispecific anti-TfR/BACE1. At trace doses, there was higher RMT and brain accumulation observed with the anti-TfR/BACE1 bispecific antibody compared to the anti-BACE1 antibody. These findings accurately fit models presented earlier in the study regarding antibody affinity, concentration, and RMT. At therapeutic doses, however, the anti-TfR/BACE1 bispecific antibody demonstrated significantly higher RMT compared to anti-BACE1 or anti-TfR alone.

To explore the cellular basis of the improvements made by Yu et al. (2011) previously, Bien-Ly et al. (2014) explored whether TfR antibody affinity alters TfR intracellular trafficking after receptor-ligand endocytosis. When researchers compared high- and low-affinity TfR bispecific antibodies, it was found that high-affinity binding to TfRs caused a dose dependent RMT and reduction of brain TfR levels. Using live imaging and colocalization experiments in vitro, researchers determined that high-affinity TfR bispecific antibodies facilitated the trafficking of TfRs to

lysosomes and thus induced the degradation of TfR, an observation which was confirmed in vivo. This ultimately led to the research team determining that high-affinity anti-TfRs alter the process of TfR trafficking and dramatically impacts the ability of TfRs to facilitate BBB transcytosis.

Similar findings regarding the influence of affinity on TfR trafficking were also found by Sade et al. (2015). In this study's in vitro model of the human BBB using human cerebral microvascular endothelial cells (hCMEC/D3), it was found that low-affinity anti-TfRs were able to transcytose across the hCMEC/D3 cells, whereas high-affinity antibodies were directed to lysosomes. This suggests vesicle trafficking may be affected by the targeting ligand used for RMT. Based on these findings, Clark and Davis (2015) examined whether Tf and anti-TfR antibodies behaved differently when used as targeting agents for nanoparticles with the addition of an acid-cleavable linkage - diamino ketal (DAK). The results of this examination indicated that high-avidity nanoparticles showed a nearly threefold increase in their ability to reach the parenchyma in vivo after the incorporation of the DAK linker. It was also observed that there existed a direct relationship between surface Tf-DAK content on the nanoparticles and their brain penetration.

DISCUSSION OF USING TRANSCYTOSIS FOR BIOLOGIC DELIVERY ACROSS THE BLOOD-BRAIN BARRIER

Several considerations should be made when examining the potential success of delivering a drug through transcytosis, the first of which is the drug's ideal environment. For example, the extent of cationization increases as the pH of the protein's environment decreases (Herve, Ghinea, and Scherrmann, 2008). Therefore, if the peptide drug is only stable in relatively basic conditions, AMT may not be effective at physiological pH. Transcytosis may also alter the function of the drug molecule. Linking cationic groups to a peptide may change its activity at the drug target meaning AMT may not be possible with all drugs (Goulatis and Shusta, 2017). There is no evidence insofar as to suggest this occurs with RMT as well, although the current developmental stage of RMT-technology may not allow comprehensive investigation of this phenomenon.

One of the most important issues with the application of AMT is that the exact mechanisms whereby directly cationized molecules, CPPs, and many receptor-bound ligands are transcytosed to the CNS remain mostly elusive (Betsholtz, 2014; Herve, Ghinea, and Scherrmann, 2008; Mäger et al., 2017). Some studies only claim that endocytosis is always involved (Yang et al., 2013), while almost all are unclear about what occurs after absorptive endocytosis is triggered (Gabathuler, 2010; Chen and Liu, 2012; Kamalinia et al, 2015). Most schematic diagrams illustrate a direct path for therapeutics molecules being internalized into the endothelial cell to exocytosing at the abluminal surface. In reality, however, vesicles are still likely to progress to a lysosomal path in AMT (Banks, 2012). Not elucidating why a vesicle may be directed towards transcytosis versus degradation may ultimately contribute to the reduction of the success of drug delivery techniques. As mentioned earlier, the transfection levels of the Tf-conjugated delivery vectors in RMT is perhaps the most difficult obstacle to overcome in the progression and development of RMT as a method of therapeutic delivery to the brain (Gosk et al. 2004; Lajoie and Shusta, 2015). Of course, the issue that arises while attempting to deliver therapeutics via RMT is the propensity for endosomes to be directed for lysosomal degradation. While research is being conducted to determine how to counter this challenge, it is still a significant limit on RMT's efficacy as a delivery pathway for biologics.

Another consideration with the use of RMT is that, depending on the method of dose administration, there are varying levels of biodistribution within the subject. This level of distribution may vary based on the species and even the individual administered the biologic, as well as based on the carrier or loaded-nanoparticle used. In several studies, antibiotic tagged nanoparticles accumulate in areas of the body other than the brain, such as the spleen, liver, kidneys, lungs, and heart of mice, and in almost all tissue of primates (Friden et al., 1996; Johnsen et al., 2017). Such a distribution of antibodies and nanoparticles is likely due to the variable level of expression of the TfRs throughout the body, since it is not exclusive to BMECs. The use of the TfRs as a means of RMT holds an innate flaw regardless of treatment since iron is required by all tissues in the body in order to collect and endocytosis diferric iron floating in the bloodstream. Similarly, due to its reliance on electrostatic interactions, many researchers believe AMT is not specific

enough for therapeutic deliver to the brain and can lead to toxic effects on other surrounding organs (Rocha, 2013; Goulatis and Shusta, 2017). However, the mechanism whereby one specific type of molecule crosses the blood-brain barrier by AMT - diamine or polyamine-modified peptides - has not yet been ascertained. Therefore, it is likely that their transport requires not just electrostatic interactions, but carrier-mediated transport as well, which may prove to be more specific (Rocha, 2013). As well, many cationized molecules were observed to have decreased half-lives, which would decrease their bioavailability in the blood. Along with no reports of cationized peptides or CPPs disrupting the BBB, delivering therapeutics through AMT appear much less traumatic and toxic for patients with neurodegenerative diseases. On the other hand, a decreased half-life may also lower the therapeutic window for a drug to a level that renders it ineffective (Yi et al., 2014). Therefore, additional studies should be conducted for each potential drug to evaluate the effect of the local environment, the linkage or transformation process itself, non-specificity, and decreased half-life on its function and activity.

It should be noted that there can be many advantages of selecting AMT as the transport pathway for a drug over RMT. First, the capacity of AMT is significantly greater than RMT. If the volume of a certain therapeutic molecule required needs to be dramatically increased, AMT is an ideal transport process, since it is not limited by the number of receptor proteins expressed on the endothelial surface (Upadhyay, 2014). The anionic and cationic properties of the molecules and membranes in question will also remain relatively consistent, ensuring that AMT is a safe choice for therapeutic delivery.

Many of the considerations for RMT discussed thus far are specific to the use of Tf and the TfRs. While RMT is an important and extensively researched method for neurological drug delivery, there are few instances in research and experimentation that demonstrate sufficient RMT to treat a neurological disease. This is perhaps largely due to the lack of data that exists for receptors that are selective and abundant on BMECs, and a focus on only a select few RMT receptors, such as the Tf and insulin receptors, their antibodies, and nanoparticles, such as liposomes (Mäger et al., 2017). This particular issue in research and literature can be resolved through the search for and study of other receptors, targeting methods, and potentially drug carrying ligands. Such research is being conducted by Mäger et al. (2017) who, with the Collaboration on the Optimization of Macromolecular Pharmaceutical Access to Cellular Targets (COMPACT) project, have set up a proteomics- and transcriptomics-based workflow to identify brain microvascular cell surface specific receptors with the aim of using them for biologic delivery across BMECs. Other receptors have the potential to become extremely specific, yet effective means through which therapeutic molecules can be delivered, with much more control and less side effects than AMT. Ultimately, the future clinical application of both AMT and RMT for the delivery of therapeutic agents to the brain must be built on a more solid foundation of fundamental knowledge and understanding.

CONCLUSION

While the exploration of AMT and RMT as potential therapeutic drug delivery mechanisms continues to advance, there is still a lack of basic conceptual understanding surrounding these processes that limits their potential for clinical success. There does appear to be progress in terms developing new agents for the treatment of neurological diseases, most pointedly the Genentech and Roche partnership and development of a stage II/III drug to treat AD, though this does not compensate for the lack of suitable current understanding and use of transcytosis for therapeutic delivery to the brain (Boettner, 2014). Continued study and development of technologies and methodologies that make use of transcytosis for brain biologic delivery must persist. AMT is an asset for increasing the therapeutic effect of drugs by reducing their toxicity in peripheral organs, as well as allowing them to successfully transcytose through the BBB. On the other hand, RMT may be able to minimize the influence that a mode of drug delivery has on the efficacy of a drug while also allowing for enhanced transcytosis of therapeutics to the brain. An additional avenue to explore is the combination of several transport pathways to create a more integrated and cohesive method of biologic delivery allowing for greater brain uptake and accumulation. Ultimately, our current understanding of the fundamental properties and mechanisms that govern AD and the BBB is insufficient for troubleshooting many drug delivery technologies that are undergoing development.

As new drugs are discovered, further investigation will also be required for methods of delivering these drugs to their intended targets as well, though AMT and RMT are both promising endogenous transport pathways that may be harnessed for this purpose in the future.

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AUTHORSHIP AND CONTRIBUTIONS

Coulter Montague was responsible for conducting a review of the literature and reporting on findings related to receptor-mediated transcytosis. Chen Chen was responsible for conducting a review of the literature and reporting on findings related to absorptive-mediated transcytosis. Both authors were responsible for examining and reporting on literature related to the blood-brain-barrier, and partnered to complete the introduction, discussion, and conclusion sections for this paper.

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