Exploiting Receptor-Mediated Transcytosis to Deliver Therapeutics to the Brain

Caitlin Blinkhorn

The Colorado College Department of Psychology

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Dr. Lori Driscoll

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On my honor, I have neither given nor received any unauthorized aid on this assignment. *CB*

Abstract

Delivering drugs to the brain has historically been a difficult task due to the impermeability of the blood-brain barrier (BBB) and the brain endothelial cells (BECs), which make up the walls of the blood vessels in the central nervous system (CNS). Despite immense efforts to circumvent the BBB, several non-invasive brain drug delivery methods have emerged, such as focused (FUS) and scanning (SUS) ultrasound, nanocarriers, and receptor-mediated transcytosis (RMT). Although these techniques have yet to be FDA approved, RMT offers a unique opportunity for drug delivery, as receptors already expressed on the BBB pose as targets for large drug complexes to enter the brain through endocytosis. One of the most targeted receptors for RMT is the transferrin receptor (TfR), which naturally transports extracellular iron to the brain by binding its natural ligand, transferrin, which carries iron molecules. In comparison to other possible BBB targets, TfR holds great promise due to its relatively consistent expression throughout age and disease progression, ability to be targeted without interfering with its normal function, and its high internalization rate in the brain. This critical literature review discusses several methods for brain drug delivery, highlighting the future potential and remaining challenges RMT faces.

Keywords: receptor-mediated transcytosis, RMT, blood-brain barrier, BBB, therapeutics, drug delivery

Go With the Flow: Exploiting Receptor-Mediated Transcytosis to Deliver Therapeutics to the Brain

The central nervous system (CNS) contains some of the body's most complex interfaces through which to deliver therapeutics. The CNS is protected by the blood-brain barrier (BBB), a highly regulated natural gatekeeper between the blood and neural tissue (Abbott, 2014). This barrier limits toxins and other pathogens from entering the brain, while selectively controlling the transport of nutrients, ions, and signaling molecules into the brain to establish and maintain homeostasis (Sweeney et al., 2019, as cited in Zhao & Zlokovic, 2020, p. 1). One of the main components of the BBB are specialized brain endothelial cells (BECs) that line the microvessels and capillaries of the brain. Other BBB components include supportive structures such as astrocyte end-feet processes, an abluminal, or brain-facing, membrane called the basement membrane, and surrounding astrocytes and pericytes (Bajracharya et al., 2021; Yu & Watts, 2013). BECs in the BBB feature tight junctions, or gaps between BECs, and associated transmembrane proteins between BECs, which highly restrict the diffusion of molecules into the brain (R. Thorne, personal communication, January 25, 2022). These tight junctions are not only more restrictive than those found in other endothelia throughout the body (Abbott, 2014), but they also lack continuous gaps and channels connecting the luminal (blood-facing) and abluminal membranes (Friden et al., 1991).

The 'closed' nature of the cerebral endothelial junctions was first observed by intravenously administering horseradish peroxidase in mice, resulting in the staining of all tissues save for the brain and spinal cord (Reese & Karnovsky, 1967). Because of the presence of these tight junctions, molecules must enter the brain through the BECs themselves through what are called transcellular pathways (Bajracharya et al., 2021). However, this movement is restricted

in BECs, making the transcellular pathway infeasible for most therapeutics (Reese & Karnovsky, 1967). Therefore, the macromolecules and nutrients which do enter the brain do so through specific pathways, including adsorptive-mediated transcytosis (AMT) for positively charged molecules (e.g., albumin, passive diffusion for small lipophilic molecules), carrier-mediated transcytosis (CMT) for small polar molecules (e.g., glucose), or receptor-mediated transcytosis (RMT) for large macromolecules (e.g., insulin and transferrin; De Bock et al., 2016, as cited in Bajracharya et al., 2021, p. 2). Therefore, to cross the BBB strategically and effectively, therapeutics for brain disorders must be developed with the challenges of these unique components in mind (Bourassa et al., 2019).

Mechanisms for circumventing the barrier

Approximately 98% of small molecule drugs and ~100% of large molecule drugs developed for brain disorders are unable to cross into the BBB (Leinenga et al., 2016). Additionally, only lipophilic drugs with a molecular weight less than ~0.4 kDa can diffuse through the barrier to an appreciable degree (Yu & Watts, 2013), and even then, these drugs often have poor brain distribution due to BBB efflux transporters, which pump drugs out of endothelial cells towards the blood (Tachikawa et al., 2014). Although invasive procedures such as intracranial, interventricular (through cerebrospinal fluid in cerebral vesicles), and intrathecal (through the spinal canal) injections are able to bypass the BBB and can lead to higher drug concentrations in the brain (Kariolis et al., 2020), they cannot be used for multiple dosing regimens, have a high cost of maintenance, and are associated with local toxicity and increased intracranial pressure, which can cause complications such as infections and brain trauma (Pandit et al., 2020). As a result, recent clinical and research efforts have focused on the development of less invasive and more effective methods to deliver therapeutics to the brain, as today the most

common routes of administration for biotherapeutic delivery are intravenous (IV) or cerebrospinal fluid (CSF) injection (Pardridge, 2020). However, most therapies for neurodegenerative diseases have failed in trials because they are not developed to cross and transport drugs across the BBB— brain drug development continues to operate in a "BBB-free zone" (Pardridge, 2020, p. 2).

As an example of this problem, one kind of therapeutic that is currently being engineered to cross the BBB are monoclonal antibodies (mAbs), laboratory-produced antibodies derived from the host's own white blood cells that uniquely bind to the same part of a certain target, or epitope (Lu et al., 2020). mAbs have become a primary treatment modality for a variety of indications such as cancer, transplant rejection, infectious diseases, and more during the past 25 years. Over the past five years, they have become the top selling class of drugs in the pharmaceutical market (Lu et al., 2020). Several key features of mAbs include high target specificity throughout the body, and the ability to target more than one epitope (i.e., bi-specific mAbs), contributing to few side and off-target effects (Buss et al., 2012).

In addition to targeting key molecules involved in brain disease pathologies, mAbs and smaller mAb fragments can be fused to a variety of therapeutic molecules to enhance their transport and uptake in the brain (Kariolis et al., 2020) after intravenous administration. For example, Friden et al. (1991) first observed increased brain uptake of methotrexate (MTX), a chemotherapy compound, in the rat brain when it was fused to the OX-26 mAb, in contrast to MTX on its own. Other advantages of mAbs include favorable pharmacokinetic (PK) characteristics such as long serum half-lives (Buss et al., 2012). In contrast, smaller molecules generally have shorter half-lives and, as a result, need to be administered daily or multiple times a day (Ovacik & Lin, 2018).

Although recent advances in antibody engineering have led to improved safety and efficacy (Lu et al., 2020), only three mAbs are currently approved for the treatment of neurological diseases, including Biogen's aducanumab for Alzheimer's disease (AD) (Bajracharya et al., 2021). Despite the clinical advantages of mAbs and their use in other diseases, mAbs are large molecules (~150 kDa), which typically cannot cross the barrier on their own in sufficient concentrations to elicit a therapeutic response in humans (Kariolis et al., 2020). For example, only 0.1%-0.2% of peripherally administered antibodies cross the BBB, most likely through non-specific fluid phase endocytosis, or micropinocytosis, where antibodies diffuse non-specifically across BECs (Yu & Watts, 2013).

Thus, recent efforts have focused on either increasing therapeutic doses or improving brain uptake of mAbs. Efforts focusing on improving brain uptake has led to the discovery of additional ways to cross the BBB (Patel & Patel, 2017), and importantly, recent research has demonstrated therapeutic potential in mice, monkeys, and humans (Kariolis et al., 2020; Leinenga et al., 2021; Ullman et al., 2020). Several techniques, including therapeutic ultrasound, nanotechnology and nanoparticles, and receptor-mediated transcytosis (RMT), have been shown to improve the brain uptake of mAbs and other molecules, which remains to be a primary challenge in the treatment of neurodegenerative disorders. Focused (FUS) and scanning (SUS) ultrasound involve the reversible, mechanical opening of the BBB to allow molecules to enter both para- and transcellularly (Pandit et al., 2020). Nanoparticles are nanoscale carrier structures such as liposomes and gold nanoparticles, which can endogenously enter the brain and either be conjugated to or encapsulate a variety of molecules including monoclonal antibodies (mAbs) (Bajracharya et al., 2021). For example, nanoparticles can enter the brain through receptor-mediated transcytosis (RMT), the third method highlighted in the present review. For RMT,

antibodies and drug complexes are designed to target receptors already expressed on BECs, which then undergo transcytosis to be released on the luminal side of the BBB and into the brain. Although other mechanisms such as intranasal or viral-mediated brain delivery continue to be investigated (Pandit et al., 2020), RMT and focused ultrasound are primarily used to improve large molecule and antibody uptake in the brain (Bajracharya et al., 2021).

Therapeutic ultrasound

Two kinds of therapeutic ultrasound— FUS, a more localized or targeted sonication, and SUS, which applies multiple sonication spots across the brain (Bajrachrya et al., 2021)— have been explored both as possible treatments for brain diseases (Leinenga et al., 2021) and to open the BBB to improve the uptake of large molecule therapeutics. Currently, FUS is currently in human clinical trials, both on its own for AD (Insightec, NCT03739905; Konofagou, NCT04118764), and in combination with the administration of other therapeutics such as mAbs (NaviFUS Corporation, NCT04446416) as a cancer treatment. The possibility of FUS to be used either on its own or alongside other therapeutics demonstrates its potential as a treatment for neurological conditions.

Therapeutic ultrasound differs from standard imaging ultrasound in two ways: it uses a lower frequency to avoid tissue heating and its effects depend on the acoustic pressure applied (Leinenga et al., 2016). In addition, therapeutic ultrasound to open the BBB requires an intravenous injection of microbubbles (MBs), which are gas-filled lipid or protein-encased shells that reduce negative thermal effects, help localize and amplify sound waves, and most importantly, produce mechanical forces on the BBB by oscillating, leading to the barrier's transient opening (Hersh et al., 2016). Even though ultrasound-induced BBB opening occurs through several different mechanisms, the overall understanding of how the BBB opens under

these conditions remains unknown (Leinenga et al., 2021). Still, it is understood that therapeutic ultrasound induces several structural and molecular changes, including decreased tight junction integrity, a subsequent increase in the vascular permeability of the BBB (Leinenga et al., 2019), increased endocytosis at the BEC surface due to the upregulation of caveolin, a transmembrane protein, and the activation of microglia and astrocytes due to an inflammatory response (Kovacs et al., 2016). However, the molecular impacts after therapeutic ultrasound remain to be investigated; data are mixed regarding the benefits and risks of such post-treatment responses.

For example, Kovacs et al. (2016) reported microbubble dose-dependent cellular and molecular disruptions indicating sterile inflammation (SIR) comparable to ischemia or traumatic brain injury (TBI), which lasted up to 24 hrs after FUS. Additionally, signs of astrocytic and early neuronal injury in mice were observed, even though this phenomenon may have a clinical benefit. Specifically, microglial and astrocyte activation from SUS-mediated BBB opening can increase phagocytosis and internalization of Aβ plaques, proteins which aggregate and contribute to cognitive decline in AD (Leinenga et al., 2018, 2021). Interestingly, when SUS was applied without the use of MBs, Leinenga et al. (2019) found no reduction of Aβ plaques, indicating the crucial role of MBs in opening the BBB.

Importantly, enough data on the safety and reversibility of FUS and SUS-mediated BBB opening have been collected in non-human models for the technique to be investigated in humans (Pandit et al., 2020). There are several on-going clinical trials investigating the effects of FUS in AD, gliomas, and other brain diseases (Insightec, NCT03739905; Konofagou, NCT04118764; Jordan, S., NCT04063514), with primary endpoints of safe, successful BBB opening and the few adverse events. The application of FUS and SUS plus MBs has been studied both with and without the additional administration of therapeutics such as mAbs, chemotherapy

agents, and nanoparticles (Pandit et al., 2020). Importantly, both techniques have shown to enhance the delivery and brain uptake of such therapeutics due to BBB opening. FUS is also undergoing clinical trials in combination with mAbs such as trastuzumab and bevacizumab, approved chemotherapeutics, for the treatment of breast cancer brain metastases (Insightec, NCT03714243) and glioblastomas, or tumors in the brain (NaviFUS Corporation, NCT04446416).

Some of the remaining limitations of therapeutic ultrasound are the lack of a universal protocol to minimize the risk of both neuronal degeneration and adverse effects, and an incomplete understanding of the technique itself and the bioeffects it induces. For example, microbubble size and dosage need to be closely monitored for optimal efficacy and safety outcomes (Konofagou, 2014; Patel & Patel, 2017). Although the safety history of FUS and SUS has drastically improved over the past decade, particularly from the additional administration of MBs, a better understanding of the relationship between technique parameters and BBB opening will be critical to guide future research and approval status (Konofagou, 2014).

Nanocarriers and nanoparticles

Nanocarriers are biocompatible or chemically modified molecules within a specific size range that can encapsulate or be conjugated to various compounds to carry them across the BBB (Gaillard et al., 2014). Nanoparticles are a class of nanocarrier characterized by their site-specific targeting and controlled drug release (Alam et al., 2010, as cited in Hersh et al., 2016, p. 1180). This site-specific targeting is primarily mediated by ligands that are fused or encapsulated by nanoparticles, which otherwise would not be able to cross the BBB. Several nanocarriers which have demonstrated efficacy for BBB drug delivery include dendrimers, gold nanoparticles, and liposomes, all of which cross the BBB through several means of transport, including RMT

(liposomes and dendrimers), CMT (dendrimers and gold nanoparticles), AMT (liposomes), and passive diffusion (gold nanoparticles) (Bajracharya et al., 2021; Hersh et al., 2016).

Dendrimers are polymeric nanoparticles defined by their organized structures and branched, tree-like monomers to which therapeutics can be conjugated (Kumar et al., 2018, as cited in Bajracharya et al., 2021, p. 9). One of the advantages of polymeric nanoparticles is their ability to be conjugated to a variety of molecules due to the many functional groups present on their large surface area (Hersh et al., 2016). For example, dendrimers have successfully crossed the BBB via transcytosis to deliver non-coding RNA, mAbs (Jin et al., 2021, as cited in Bajracharya et al., 2021), and a variety of other molecules to the brain. Remaining limitations of polymeric nanoparticles are toxicity- and safety-related concerns; due to the range of endpoints and polymers used in research, it is challenging to compare the safety and toxicity profiles of various nanoparticle formations (Gaillard et al., 2014).

Other well-researched nanocarriers are liposomes, self-assembled vesicles composed of lipid bilayers, which are unique in their ability to carry hydrophobic or hydrophilic compounds across the BBB via either RMT or AMT and without modification to the compound (Gaillard et al., 2014; Hersh et al., 2016). Liposomes further differentiate themselves from other nanoparticles by their biocompatibility, biodegradability, and low toxicity (Gaillard et al., 2014). Like other nanoparticles, most nontargeted liposomes do not cross the BBB in sufficient quantities and are commonly pegylated or coated with additional molecules (Gaillard et al., 2014), which can then facilitate movement to the brain through one of the transcellular pathways. For example, Das et al. (2020) found that gold-rod coated, light-activated liposomes could be melted by laser stimulation, inducing the reversible ‘melting’ of lipid bilayers and subsequently the controlled and repetitive release of MTX. This discovery holds importance for the use of

liposomes in brain diseases due to light-triggering manipulation, which could be applied to specific areas of the brain for on-demand drug delivery (Das et al., 2020).

A defining property of nanoparticles, another kind of nanocarrier made of either gold, silver, or iron oxide (Hersh et al., 2016) is that they are generally smaller than liposomes and, as a result, cannot encapsulate drug compounds. Nonetheless, a variety of agents such as antibodies and chemotherapeutics can be conjugated to the surface of metallic nanoparticles, which can then cross the BBB via RMT or passive transmembrane diffusion (Bajracharya et al., 2021). For example, Li et al. (2021) conjugated antibodies targeting tight junction proteins to gold nanoparticles (AuNPs), and through the laser-stimulation of these nanoparticles, were able to induce a graded and reversible increase in BBB permeability. The increase in brain uptake of antibodies, liposomes, and viral vectors was due to an increase in paracellular diffusion from the widening of tight junctions, and importantly, was restricted to only where the laser stimulation was applied (Li et al., 2021).

However, there are conflicting data regarding the efficacy of transporting nanoparticles conjugated with antibodies via RMT. Specifically, Cabezón et al. (2015) found that, although most AuNPs conjugated to TfR-targeting antibodies underwent RMT, very few were found on the abluminal membrane and in the brain parenchyma of mice, suggesting the AuNPs were not released from the BECs, potentially due to endosomal sorting. Although RMT-based drug delivery remains one of the most promising drug delivery strategies, the efficiency of the nanoparticle transcytosis containing the drug ‘cargo’ needs to be improved (Cabezón et al., 2015). Antibody drug-complexes without nanoparticles designed for RMT have also faced this challenge, but this has mostly been addressed by lowering the affinity to which the anti-TfR antibody binds to TfR (Yu et al., 2011).

Despite evidence demonstrating the efficacy of nanoparticles in delivering antibodies and other molecules across the BBB, it is important to highlight several other limitations. For example, several studies have demonstrated long-term toxicity and immunological safety responses after gold and other metallic nanoparticle treatments (Bajracharya et al., 2021; Gaillard et al., 2014). Additionally, anomalous complement activation, a part of the human immune system, has been observed after intravenous administration of either liposomes or dendrimers, and continues to be a safety concern affecting both nanocarriers (Gaillard et al., 2014). Jiskoot et al. (2014) also observed other immunological risks such as antibody formation against certain nanoparticle components, which led to reduced efficacy, bioavailability, and increased toxicity of the nanocarrier (as cited in Gaillard et al., 2014, p. 447).

Furthermore, unlike therapeutic ultrasound, which is currently being studied in human AD research (Kaplitt et al., 2018), only a handful of nanoparticles focused on neurodegenerative conditions have reached clinical trials, and importantly, all have failed (Wolfram et al., 2015, as cited in Bajracharya et al., 2021, p. 8). Currently, there are very few nanoparticles in clinical trials for brain diseases besides brain cancer and gliomas. Nonetheless, preclinical data have demonstrated the therapeutic potential of nanoparticles and other nanocarriers for BBB drug delivery, and future research should be guided by these safety indications.

Receptor-mediated transcytosis (RMT)

Exploiting endogenous transport systems, such as RMT (Yu & Watts, 2013), may be the most promising avenue for overcoming the low brain uptake of large molecules such as antibodies. RMT is a highly specific approach that minimizes off-target and other side-effects and has demonstrated improved brain uptake of a variety of therapeutic molecules, including enzymes, antibodies, and chemotherapeutic agents, highlighting its ability to potentially treat

numerous kinds of neurological diseases (Friden et al., 1991; Kariolis et al., 2020; Niewoehner et al., 2014; Ullman et al., 2020). Like most other organs in the body, the brain depends on nutrients, growth factors, and signaling peptides for normal growth and function (Friden et al., 1991), and it acquires these essential molecules through transcellular transport systems such as RMT, CMT, and AMT. These endogenous transport systems highlight the fact that the bloodstream and the brain are not completely isolated from one another (Bourassa et al., 2019). RMT exploits natural transport systems and specific receptors expressed on the BBB to deliver drugs to the brain in three primary steps: 1) the endocytosis of a receptor-ligand complex on the blood or luminal side of the endothelial cell, followed by 2) the transport of the complex across the cytoplasm, and 3) exocytosis to the abluminal or brain side of the cell (Gabathuler, 2010).

Protein engineering techniques have led to the ability for therapeutics to be fused to receptor-targeting mAb therapeutics to cross the BBB via RMT and improve brain uptake in contrast to the therapeutic alone (Pandit et al., 2020). Specifically, the mAb-drug complex, deemed a molecular Trojan horse, ‘sneaks’ the fused therapeutic into the brain by acting as an endogenous ligand to be subsequently internalized and releases the drug into the brain. For example, Hultqvist et al. (2017) found, in mice, an 80-fold concentration increase in an antibody targeting Aβ precursor molecules (mAb158) that were fused to a TfR antibody in comparison to the unmodified mAb158. Further, RMT has been reported to increase the brain delivery of mAbs by 2-3% of the injected dose (Bajracharya et al., 2021), a significant increase from the 0.1% of peripherally administered antibodies that have been reported to cross the BBB on their own (Yu & Watts, 2013).

RMT targets. Numerous proteins expressed on the BBB can be targeted for RMT, including the human insulin receptor (HIR) and the erythropoietin receptor (EPOR). Boado et al.

(2010) simultaneously targeted both receptors in primates with a molecular Trojan horse consisting of a chimeric mAb fused to erythropoietin (EPO). EPO is a neurotrophic factor that has the potential to be developed as a treatment for neurodegenerative diseases such as Parkinson's (Boado et al., 2010). By designing a HIRMAb-EPO fusion protein with high affinities for both EPOR and HIR, significant levels of EPO were rapidly and selectively transported into the brain (Boado et al., 2010).

However, chronic HIRMAb treatment may negatively affect hematopoiesis in organs such as the heart (Boado et al., 2010), as insulin receptors are importantly involved in maintaining glucose homeostasis (Patel & Patel, 2017) and, as a result, may not be the safest RMT target. For example, monkeys treated with a mAb fusion protein targeting the HIR experienced myocarditis, pancreatic lesions, and other adverse reactions, as the insulin receptor is also found on vascular endothelial cells and duct cells of the pancreas (Ohshima-Hosoyama et al., 2012). Furthermore, it has been hypothesized that the transport of insulin may be affected by age, which would most likely influence the efficacy of HIR-mediated RMT (Sartorius et al., 2015, as cited in Bourassa et al., 2019, p. 585). Zuchero et al. (2016) found unappreciable brain uptake of antibodies that targeted insulin, in comparison to TfR-targeted antibodies, aligning with their other findings that TfR was the highest single-pass transmembrane protein expressed on BECs and might serve as a better RMT target than HIR.

The transferrin receptor (TfR), a transmembrane protein first discovered in the 1980s using peroxidase immunohistochemistry and a mAb, OX-26 (Jefferies et al., 1984), is one of the most abundant and well-studied BBB receptors for RMT. TfR's spatial distribution and abundance on the luminal surface of the BBB make it a promising target for RMT. TfR is expressed on both the abluminal and luminal sides of the BBB (Pardridge, 2020) and is a

bidirectional receptor responsible for the influx of halo-transferrin (or transferrin that is bound to iron) and the efflux of apo-transferrin (not bound to iron) (Fishman et al., 1987; Skarlatos et al., 1995; Zhang & Pardridge, 2001, as cited in Pardridge, 2020, p. 6). Transferrin, the endogenous ligand of TfR, binds to extracellular iron and importantly acts as an iron carrier to the brain; without the influx of extracellular iron, oxygen transport, redox reactions, and other processes crucial for homeostasis would not occur (Kariolis et al., 2020). In addition, TfR is highly expressed on the brain endothelial cells, and much less so than on microvessels of other tissues, which is beneficial in attempting to prevent drug engagement in other parts of the body (Jefferies et al., 1984, as cited in Friden et al., 1991, p. 4771).

Fishman et al. (1987) was one of the first groups to find that radiolabeled transferrin is internalized and transcytosed into the brain through TfR. Friden et al. (1991) built on these findings to discover that other large molecules, such as antibodies targeting TfR, may be able to serve as drug carriers to the brain (Friden et al., 1991). For example, TfR-mediated RMT was demonstrated to increase brain concentrations of MTX when MTX was fused with OX26 (Friden et al., 1991). Unfortunately, although a specific set of TfR receptors are expressed on the BBB, TfR is also expressed in other parts of the body (R. Krishna, personal communication, December 16, 2021), even though there is insufficient information on the other locations of TfR expression in humans (Ponka & Lok, 1999). For example, one study found detectable TfR levels in the human heart, kidney, liver, spleen, bone marrow, and red blood cells (RBCs), although when red blood cells (RBCs) mature, TfRs are released, or ‘shed’ from the cell (Gatter et al., 1983, as cited in Ponka & Lok, 1999, p. 1123). The expression of TfR in other parts of the body constitute research and clinical challenges: Yu and Watts (2013) highlight that one reason why many studies have found a lack of broad brain distribution of anti-TfR antibodies is due to the

expression of TfR on peripheral organs, which leads to target-mediated clearance and a reduced amount of antibody that can be delivered to the brain.

It is possible that the therapeutic potential of RMT can be improved through engineered antibody adjustments and dosage protocols. Couch et al. (2013) found a significant reduction of young blood cells, or reticulocytes, in mice dosed with anti-TfR/BACE1 and observed acute clinical responses consistent with shock and hemoglobinuria. However, both responses were mitigated after lowering the antibody's affinity to TfR and introducing mutations that eliminated the effector function of the antibody, including complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity (Couch et al., 2013). Furthermore, after weekly doses of anti-TfR/BACE1 over the course of 4 weeks, the group found a sustained reduction in Aβ brain concentrations, a regenerative reticulocyte response, and no evidence of damage in tissues.

It is also encouraging that there appear to be some species differences in the specificity of TfR expression in favor of humans and other primates. Couch et al. (2013) investigated differences in TfR expression on circulating reticulocytes in the blood of mice, monkeys and humans, and found that monkeys and humans had essentially no TfR+ circulating reticulocytes. This finding indicates that the location of RBC maturation may differ between species: RBCs in mice mature in the blood, while those in monkeys and humans mature in the bone marrow (Couch et al., 2013). Thus, because most reticulocytes that still express TfR remain in the bone marrow, findings have demonstrated that primates are significantly less susceptible to the safety risks and loss of reticulocytes observed in mice (Couch et al., 2013; Yu et al., 2014). Furthermore, Yu et al. (2014) found that primates also dosed with anti-TfR/BACE1 had no loss

of reticulocytes, confirming that reticulocytes in primates express significantly lower TfR than those in mice.

Regarding TfR expression in humans, a significant therapeutic advantage of TfR is its stable expression over an individual's lifetime (R. Thorne, personal communication, January 25, 2022). For example, Bien-Ly et al. (2015) found no differences in TfR levels in human AD post-mortem tissue compared to age-matched control and confirmed AD tissue, demonstrating how the constant expression of TfR throughout disease progression and age make it an unrestricted and reliable target for TfR-based therapeutics. Similarly, brain uptake of a TfR-binding mAb was consistent for 12-, 18-, and 22-month-old AD mice, indicating that TfR-mediated uptake is not impaired by AD pathology or aging (Bourassa et al., 2019).

Two additional characteristics of TfR that make it an attractive target include a high rate of constitutive endocytosis, meaning the receptor is highly internalized into the cell regardless of ligand binding, and the maintenance of the natural receptor function when the receptor is bound by the therapeutic, which is critical for the brain to still obtain required amounts of transferrin and iron for normal functioning (R. Thorne, personal communication, January 25, 2022). When natural transferrin binds, it interacts with only the protease-like and helical domains of the receptor, leaving the apical domain available to be targeted by the drug-complex. As a result, there is little concern regarding TfR-mediated RMT interfering with iron transport, as TfR can continue to do its usual work while still facilitating the transport of therapeutics into the brain. Additionally, while new RMT targets continue to be investigated (Zuchero et al., 2016), TfR's long history and growing abundance of research has made it one of the most common RMT targets.

Despite some safety concerns in mice regarding TfR-mediated RMT, a novel anti-TfR mAb was recently developed and is currently in two separate Phase I/II clinical trials in children with Hunter syndrome, a rare, inherited neurodegenerative disorder which typically presents between ages 2-4, and adults with frontotemporal dementia (Denali Therapeutics, Inc., NCT04251026; Koch, NCT05262023). There are several unique characteristics of this mAb, coined a transport-vehicle (TV), including its ability to transport a variety of large molecules, such as other antibodies (antibody transport-vehicle; ATV), enzymes (ETV), and large proteins (PTV) across the BBB. The "real beauty" of the TV platform is the specifically engineered Fc (fragment crystallized) region of the antibody which binds to TfR, rather than the Fv (fragment variable) region, which historically has been where most anti-TfR antibodies bind TfR (R. Krishna, personal communication, December 16, 2021).

This TV platform has been uniquely engineered to have the TfR-binding site in the Fc region, which has allowed it to be fused to and deliver many different large molecules across the BBB while still targeting TfR (M. Kariolis, personal communication, January 12, 2022). For example, in the Hunter syndrome trial, although previous research efforts discovered how to recombinantly produce the iduronate-2-sulfatase (IDS) enzyme missing in syndrome, the greatest challenge has been the delivery of the enzyme into the brain and spinal cord. Despite data readouts from the ETV Hunter Syndrome trial in July 2021, which indicated an increase, instead of a hopeful decrease, in a neurofilament biomarker (Armstrong, 2021), more recent readouts have been positive, offering biomarker proof of concept for delivering the lacking enzyme to the brain, and supporting the initiation of a Phase 2/3 study later in 2022 (Denali Therapeutics Announces Continued Progress in DNL310 [ETV:IDS] Program for MPS II [Hunter Syndrome] Supporting Planned Initiation of Phase 2/3 Clinical Trial).

In contrast to AD, dementia, and other 'classic' neurodegenerative diseases, which are typically more genetically complex, Hunter syndrome is a monogenic disease, making it an 'easier' disease than AD to treat (R. Thorne, personal communication, January 24, 2022). Nonetheless, other companies have developed similar TVs, or 'brain shuttles,' via TfR-mediated RMT, including those targeting amyloid in the brain for the treatment of AD, and are also in Phase I/II trials (Hoffmann-La Roche, NCT04639050). Additionally, a Phase I/II trial for the PTV for frontotemporal dementia was launched by Denali Therapeutics in March 2022, highlighting their TV's therapeutic potential to treat other neurodegenerative conditions.

Although the preclinical success of the ETV and PTV have translated into their respective clinical trials, Kariolis et al. (2020) also reported the success of an ATV in mouse models of AD. The ATV was comprised of an anti-TfR Fc fragment fused to another antibody fragment targeting anti-β-secretase (BACE1), an enzyme involved in the Aβ production pathway, and demonstrated reduced brain and CSF Aβ concentrations in both mice and monkeys (Kariolis et al., 2020). Furthermore, in the same study, the ATV platform was redesigned to include two bispecific fragments that targeted BACE1 and Tau (ATV:BACE1/Tau) respectively, which both lead to Aβ plaque and neurofibrillary tangle formation in AD (Kariolis et al., 2020).

Future of TV platform and RMT for brain drug delivery

One of the most challenging aspects of CNS drug development continues to be safety (Gaillard et al., 2014). RMT, therapeutic ultrasound, and nanoparticle technology all still warrant certain safety concerns, even though they have demonstrated sufficient preclinical data to transition into human clinical trials. Particularly for biotherapeutics such as mAbs, toxicity in humans is a top concern (R. Thorne, personal communication, January 24, 2022). More safety and toxicity data should be collected on TfR-based therapeutics, especially in humans, to guide

protein engineering efforts to offer safer and more effective biologic therapies (Couch et al., 2013).

Despite both supportive data and an optimistic outlook on Denali's TV, it still may be too early to determine whether it is the ultimate solution for delivering therapeutics to the brain (Zhao & Zlokovic, 2020). Another RMT-specific challenge is whether structural disruptions in the BBB occur during disease progression (Bien-Ly et al., 2015). Several reports have suggested that changes in BBB function and/or integrity are part of AD progression, yet whether this phenomenon impacts the efficiency of TfR-mediated RMT continues to be researched (Bourassa et al., 2019). For example, in mice models of AD, researchers found that TfR-mediated RMT was not influenced by disease progression, and that human TfR levels were comparable in the brains of age-matched controls, early AD, and confirmed AD samples, further supporting TfR as the best RMT target (Bien-Ly et al., 2015; Bourassa et al., 2019).

One other significant challenge of brain drug delivery and RMT is that the diseases which it may be able to treat are still misunderstood. For example, the main drivers of AD are still unknown, and there are only a limited number of ways to test novel therapeutics and the BBB in a clinical setting, in comparison to other fields such as oncology (M. Kariolis, personal communication, January 12, 2022), where the mechanisms are well known. Despite this lack of knowledge, CNS drug developers have continued to develop therapeutics and biologics without BBB delivery strategies, leading to many failed clinical trials and currently only one disease-modifying drug that has been FDA approved (aducanumab) (Pardridge, 2020). It was not until the development of Denali's TV platform that a recombinant protein had purposefully been engineered to enable BBB transport (Pardridge, 2020). The lack of effort in BBB drug delivery technology needs to be addressed, as CNS drug developers continue to practice BBB avoidance

strategies, including drug injection directly into the brain or the CSF, as well as reporting CSF drug concentration as an index of drug transfer across the BBB (Pardridge, 2020). Although drug delivery to the brain has been a long-fought, clinical challenge, RMT has inspired the development of TfR-targeting TVs which have been designed for both BBB transport and to improve the brain uptake of therapeutics safely and effectively.

In addition, more direct comparisons of different methods of CNS drug delivery are needed to identify the most beneficial strategy or strategies. For example, Leinenga et al. (2021) assessed the effects of Aducanumab, currently the only FDA-approved, disease-modifying treatment for AD, scanning ultrasound (plus microbubbles), and both techniques together on Aβ plaques and behavior in mice. Surprisingly, only the combination treatment yielded statistically significant decreases in total plaque area compared to sham controls, and the same combination treatment group showed vast improvements in spatial memory (Leinenga et al., 2021), demonstrating that certain limitations of drug delivery strategies on their own may be overcome through these kinds of combinations (Bajaracharya et al., 2021).

In conclusion, more research specifically focused on BBB transport is warranted to improve the uptake of biotherapeutics in CNS. Future efforts for the treatment of neurodegenerative and other neurological diseases should focus on two main pillars: one involving the greater focus and improvement on BBB drug delivery, and another expanding the current knowledge of neurodegenerative diseases such as AD. RMT, and TfR-mediated RMT, is one technique which stands out by addressing the first pillar. Nonetheless, the use of TfR-targeted RMT in clinical trials for multiple neurodegenerative diseases confirms the therapeutic potential of the TV-platform, and it should continue to be investigated to assist patients in need.

References

Abbott, N. J. (2014). Anatomy and physiology of the blood-brain barriers. In M. Hammarlund-Udenaes, C. M. E. de Lange, & G. R. Thorne (Eds.), *Drug delivery to the brain: Physiological concepts, methodologies and approaches* (Vol. 10, pp. 3-22). Springer Link. 10.1007/978-1-4614-9105-7.

Armstrong, A. (2021, July 26). *Denali's Hunter syndrome data fail to impress, but 'Aduhelm-lenient' FDA could still approve*. Fierce Biotech. https://www.fiercebiotech.com/biotech/denali-s-hunter-syndrome-data-fails-to-impress-but-aduhelm-lenient-fda-could-still-approve.

Bajracharya, R., Caruso, A. C., Vella, L. J., Nisbet, R. M. (2021). Current and emerging strategies for enhancing antibody deliver to the brain. *Pharmaceutics, 13*(12), 1-16. https://doi.org/10.3390/pharmaceutics13122014.

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*(2), 289-97. https://doi.org/10.1016/j.neuron.2015.09.036.

Boado, R. J., Hui, E. K-W., Lu, J. Z., & Pardridge, W. M. (2010). Drug targeting of erythropoietin across the primate blood-brain barrier with an IgG molecular Trojan horse. *The Journal of Pharmacology and Experimental Therapeutics, 333*(3), 961-969. http://dx.doi.org/10.1124/jpet.109.165092.

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. *Molecular Pharmaceutics, 16*, 583-594. https://doi.org/10.1021/acs.molpharmaceut.8b00870.

Buss, N. A. P. S., Henderson, S. J., McFarlane, M., Shenton, J. M., & de Haan, L. (2012). Monoclonal antibody therapeutics: history and future. *Current Opinion in Pharmacology, 12*(5), 615-622. https://doi.org/10.1016/j.coph.2012.08.001.

Cabezón, I., Manich, G., Martín-Venegas, 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. *Molecular Pharmaceutics, 12*(11), 4137-4145. https://doi.org/10.1021/acs.molpharmaceut.5b00597.

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., Scearce-Levie, K.; Watts, R. J. (2013). Addressing safety liabilities of TfR bispecific antibodies that cross the blood-brain barrier. *Science Translational Medicine, 5*(183), 1-12. https://doi.org/10.1126/scitranslmed.3005338.

Das, S., Lazenby, R. A., Yuan, Z., White, R. J., & Park, Y. C. (2020). Effect of laser irradiation on reversibility and drug release of light-activatable drug encapsulated liposomes. *Langmuir, 36*(13), 3573-3582. https://doi.org/10.1021/acs.langmuir.0c00215.

Denali Therapeutics Announces Continued Progress in DNL310 [ETV:IDS] Program for MPS II [Hunter Syndrome] Supporting Planned Initiation of Phase 2/3 Clinical Trial. (2022, February 10). *BioSpace.* https://www.biospace.com/article/releases/denali-therapeutics-announces-continued-progress-in-dnl310-etv-ids-program-for-mps-ii-hunter-syndrome-supporting-planned-initiation-of-phase-2-3-clinical-trial/.

Denali Therapeutics, Inc. (2020, July 16 – 2027, July). A study of DNL310 in pediatric participants with Hunter syndrome. Identifier NCT04251026. https://clinicaltrials.gov/ct2/show/NCT04251026?term=denali+therapeutics&draw=2&rank=1.

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. *Journal of Neuroscience Research, 18*(2), 299-304. https://doi.org/10.1002/jnr.490180206.

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. *Proceedings of the National Academy of Sciences, 88*(11), 4771-4775. https://doi.org/10.1073/pnas.88.11.4771.

Gabathuler, R. (2010). Approaches to transport therapeutic drugs across the blood-brain barrier to treat brain diseases. *Neurobiology of Disease, 37*(1), 48-57. https://doi.org/10.1016/j.nbd.2009.07.028.

Gaillard, P. J., Visser, C. C., de Boer, M., Appeldoorn, C. C. M., & Rip, J. Blood-to-brain drug delivery using nanocarrier. (2014). In M. Hammarlund-Udenaes, C. M. E. de Lange, & G. R. Thorne (Eds.), *Drug delivery to the brain: Physiological concepts, methodologies and approaches* (Vol. 10, pp. 433-454). Springer Link. 10.1007/978-1-4614-9105-7.

Hersh, D. S., Wadajkar, A. S., Roberts, N. B., Perez, J. G., Connolly, N. P., Frenkel, V., Winkles, J. A., Woodworth, G. F., & Kim, A. J. (2016). Evolving drug delivery strategies to overcome the blood brain barrier. *Current Pharmaceutical Design, 22*(9), 117-1193. https://doi.org/10.2174/1381612822666151221150733.

Hoffmann-La Roche. (2021, March 15 – 2024, October 3). Brainshuttle AD: A multiple ascending dose study to investigate the safety, tolerability, pharmacokinetics, and pharmacodynamics of RO7126209 following the intravenous infusion in participants with prodromal or mild to moderate Alzheimer's disease. Identifier NCT04639050. https://clinicaltrials.gov/ct2/show/NCT04639050.

Hultqvist, G., Syvanen, S., Fang, X. T., Lannfelt, L., & Sehlin, D. (2017). Bivalent brain shuttle increases antibody uptake by monovalent binding to the transferrin receptor. *Theranostics, 7*(2), 308-218. http://dx.doi.org/10.7150/thno.17155.

InSightec, Focused Ultrasound Foundation. (2018, December 6 – 2022, December). ExAblate blood-brain barrier opening for treatment of Alzheimer's disease. Identifier NCT03739905. https://clinicaltrials.gov/ct2/show/NCT03739905.

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. https://doi.org/10.1038/312162a0.

Jordan, S., Neurological Associates of West Los Angeles. (2021, June 11 – 2027, January 20). The use of focused ultrasound and DCE K-trans imaging to evaluate permeability of the blood-brain barrier. Identifier NCT04063514. https://clinicaltrials.gov/ct2/show/NCT04063514.

Kaplitt, M., Krishna, V., & Rezai, A. R. (2018, September 18 – 2024, December). ExAblate blood-brain barrier (BBB) disruption for the treatment of Alzheimer's disease. Identifier NCT03671889. https://clinicaltrials.gov/ct2/show/NCT03671889?term=NCT03671889&rank=1.

Kariolis, M.S., Wells, R. C., Getz, J. A., Kwan, W., Mahon, C. S., Tong, R., Kim, D. J.,

Srivastava, A., Bedard, C., Henne, K. R., Giese, T., Assimon, V. A, Chen, X., Zhang, Y., Solanoy, H., Jenkins, K., Sanchez, P. E., Kane, L., Miyamoto, T.; Zuchero, Y. J. Y. (2020). Brain delivery of therapeutic proteins using an Fc fragment blood-brain barrier transport vehicle in mice and monkeys. *Science Translational Medicine, 12*(545). https://doi.org/10.1126/scitranslmed.aay1359.

Koch, Annelize. (2022, March 2 – 2025, November). A study to evaluate the safety, tolerability, pharmacokinetics, and pharmacodynamics of DNL593 in healthy participants and participants with frontotemporal dementia (FTD-GRN). Identifier NCT05262023. https://clinicaltrials.gov/ct2/show/NCT05262023?term=NCT05262023&draw=2&rank=1

Konofagou, E. E. (2014). Emerging engineering technologies for opening the BBB. In M. Hammarlund-Udenaes, C. M. E. de Lange, & G. R. Thorne (Eds.) (Eds.), *Drug delivery to the brain: Physiological concepts, methodologies and approaches* (Vol. 10, pp. 545-566). Springer Link. 10.1007/978-1-4614-9105-7.

Kovacs, Z. I., Kim, S., Jikaria, N., Qureshi, F., Milo, B., Lewis, B. K., Bresler, M., Burks, S. R., & Frank, J. A. (2016). Disrupting the blood-brain barrier by focused ultrasound induces sterile inflammation. *Proceedings of the National Academy of Sciences*, *114*(1), E75-E84. https://doi.org/10.1073/pnas.1614777114.

Kuo-Chen, W. (2020, July 18 – 2022, December). Efficacy and safety of NaviFUS system add-on Bevacizumab (BEV) in recurrent GBM patients. Identifier NCT04446416. https://clinicaltrials.gov/ct2/show/NCT04446416

Leinenga, G., Langton, C., Nisbet, R., & Götz, J. (2016). Ultrasound treatment of neurological diseases—current and emerging applications. *Nature Reviews Neurology, 12*, 161-174.

https://doi.org/10.1038/nrneurol.2016.13.

Leinenga, G., & Götz, J. (2016). Safety and efficacy of scanning ultrasound treatment in aged APP23 mice. *Frontiers in Neuroscience, 12*, 1-10. https://dx.doi.org/10.3389%2Ffnins.2018.00055.

Leinenga, G., Koh, W. K., & Götz, J. (2019). Scanning ultrasound in the absence of blood-brain barrier opening is not sufficient to clear β-amyloid plaques in the APP23 mouse model of Alzheimer's disease. *Brain Research Bulletin, 153*, 8-14. https://doi.org/10.1016/j.brainresbull.2019.08.002.

Leinenga, G., Koh, W. K., & Götz, J. (2021). A comparative study of the effects of Aducanumab and scanning ultrasound on amyloid plaques and behavior in the APP23 mouse model of Alzheimer disease. *Alzheimer's Research & Therapy, 13*(76). https://doi.org/10.1186/s13195-021-00809-4.

Li, X., Vemireddy, V., Cai, Q., Xiong, H., Kang, P., Li, X., Giannotta, M., Hayenga, H. N., Pan, E., Sirsi, S. R., Mateo, C., Kleinfeld, D., Greene, C., Campbell, M., Dejana, E., Bachoo, R., & Qin, Z. (2021). Reversibly modulating the blood-brain barrier by laser stimulation of molecular-targeted nanoparticles. *Nano Letters, 21*(22), 9805-9815. https://doi.org/10.1021/acs.nanolett.1c02996.

Lu, R.-M., Hwang, Y.-C., Liu, I.-J., Lee, C.-C., Tsai, H.-Z, Li, H.-J., & Wu, H.-C. (2020). Development of therapeutic antibodies for the treatment of diseases. *Journal of Biomedical Science, 27*(1), 1-30. https://doi.org/10.1186/s12929-019-0592-z.

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*(1), 49-60. https://doi.org/10.1016/j.neuron.2013.10.061.

Ohshima-Hosoyama, S., Simmons, H. A., Goecks, N., Joers, V., Swanson, C. R., Bondarenko, V., Velotta, R., Brunner, K., Wood, L. D., Hruban, R. H., & Emborg, M. E. (2012). A monoclonal antibody-GDNF fusion protein is not neuroprotective and is associated with proliferative pancreatic lesions in Parkinsonian monkeys. *PLOS One, 7*(6). https://doi.org/10.1371/journal.pone.0039036.

Ovacik, M., & Lin, K. (2018). Tutorial on monoclonal antibody pharmacokinetics and its considerations in early development. *Clinical and Translational Science*, *11*(6), 540–552. https://doi.org/10.1111/cts.12567.

Pandit, R., Chen, L., & Götz, J. (2020). The blood-brain barrier: Physiology and strategies for drug delivery. *Advanced Drug Delivery Reviews, 165-166*, 1-14. https://doi.org/10.1016/j.addr.2019.11.009.

Pardridge, W. M. (2020). Blood-brain barrier and delivery of protein and gene therapeutics to brain. *Frontiers in Aging Neuroscience, 11*, 1-27. https://doi.org/10.3389/fnagi.2019.00373.

Patel, M. M., & Patel, B. M. (2017). Crossing the blood-brain barrier: Recent advances in drug delivery to the brain. *CNS Drugs, 31*(2), 109-133. http://dx.doi.org/10.1007/s40263-016-0405-9.

Ponka, P., & Lok, C. N. (1999). The transferrin receptor: Role in health and disease. *The International Journal of Biochemistry & Cell Biology, 31*(10), 1111-1137. https://doi.org/10.1016/S1357-2725(99)00070-9.

Reese, T. S., & Karnovsky, M. J. (1967). Fine structural localization of a blood-brain barrier to exogenous peroxidase. *The Journal of Cell Biology, 34*(1), 207-217. https://doi.org/10.1083/jcb.34.1.207.

Skarlatos, S., Yoshikawa, T., & Pardridge, W. M. (1995). Transport of [^{125}I]transferrin through the rat blood-brain barrier. *Brain Research, 683*(2), 164-171. https://doi.org/10.1016/0006-8993(95)00363-u.

Tachikawa, M., Uchida, Y., Ohtsuki, S., & Teraski, T. (2014). Recent progress in blood-brain barrier and blood-CSF barrier transport research: Pharmaceutical relevance for drug delivery to the brain. In M. Hammarlund-Udenaes, C. M. E. de Lange, & G. R. Thorne (Eds.), *Drug delivery to the brain: Physiological concepts, methodologies and approaches* (Vol. 10, pp. 23-62). Springer Link. 10.1007/978-1-4614-9105-7.

Ullman, J. C., Arguello, A., Getz, J. A., Bhalla, A., Mahon, C. S., Wang, J., Giese, T., Bedard, C., Kim, D. J., Blumenfeld, J. R., Liang, N., Ravi, R., Nugent, A. A., David, S. S., Ha, C., Duque, J., Tran, H. L., Wells, R. C., Lianoglou, S.; Henry, A. G. (2020). Brain delivery and activity of a lysosomal enzyme using a blood-brain barrier transport vehicle in mice. *Science Translational Medicine, 12*(545). https://doi.org/10.1126/scitranslmed.aay1163.

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. *Science Translational Medicine, 3*(84). https://doi.org/10.1126/scitranslmed.3002230.

Yu, Y. J., & Watts, R. J. (2013). Developing therapeutic antibodies for neurodegenerative disease. *Neurotherapeutics, 10*(3), 459-472. https://doi.org/10.1007/s13311-013-0187-4.

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., Scearce-Leavie, K., Couch, J. A., Dennis, M. S.; Watts, R. J. (2014). Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates. *Science Translational Medicine, 6*(261), 1-8. https://doi.org/10.1126/scitranslmed.3009835.

Zhao, Z., & Zlokovic, B. V. (2020). Therapeutics TVs for crossing barriers in the brain. *Cell, 182*(2), 267-269. https://doi.org/10.1016/j.cell.2020.06.041.

Zuchero, Y. J. Y., Chen, X., Bien-Ly, N., Bumbaca, D., Tony, 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*(1), 70-82. https://doi.org/10.1016/j.neuron.2015.11.024.