DELIVERY AND THE BRAIN BARRIERS

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INTRODUCTION

Delivery of therapeutic agents to the brain is limited by the presence of the Blood-Brain Barrier (BBB). Despite great strides in the basic science of brain physiology and disease in the past decade, delivery issues have received minimal attention. Current estimates are that 98% of all small molecule drugs minimally cross the BBB, and miniscule amounts of large molecule drugs cross the BBB, except leakage in areas of BBB dysfunction. This disconnect has slowed the application of pharmacotherapy and immunotherapy in brain diseases. In this report we review the major advances in brain drug targeting research in the last 5 years, including approaches to circumvent the BBB for brain delivery by making use of endogenous transport mechanisms or bypassing the BBB altogether. We also discuss the major unresolved problems in brain drug targeting, barriers to progress and important future areas of research.

RECENT ADVANCES IN BRAIN DRUG DELIVERY RESEARCH:

1. Receptor-mediated transport (RMT).

The BBB expresses RMT systems for the transport of endogenous peptides, such as insulin or transferrin. The RMT systems operate in parallel with the classical carrier-mediated transporters (CMT), which transport certain small molecule nutrients, vitamins, and hormones. Just as the CMT systems are portals of entry for small molecule drugs that have a molecular structure that mimics that of an endogenous CMT substrate, the RMT systems are portals of entry for large molecule drugs that are attached to endogenous RMT ligands.

a. Monoclonal antibody (MAb) molecular Trojan horses (MTH). Genetic engineering is used to produce either chimeric or humanized forms of the monoclonal antibody.[1,2] The most potent antibody-based MTH known to date is monoclonal antibody against the human insulin receptor.[3] Recently, this antibody has been humanized, and shown to cross the BBB in vivo in non-human primates.[2] Certain peptidomimetic MAbs act as ligands for the RMT systems. These BBB RMT-specific antibodies bind epitopes on the receptor which are spatially removed from the endogenous ligand binding site. The peptidomimetic MAbs act as MTH to ferry across the BBB an attached drug, protein, antisense agent, or non-viral plasmid DNA.[4-7] A number of non-

antibody delivery systems have been evaluated, including histone,[8] p97,[9] receptor-associated protein (RAP),[10] the tat transduction domain peptide,[11] and other cationic peptides or polymers.[12] Whereas the transport of ligands such as RAP is hypothesized to be receptor-mediated,[10] the transport of cationic peptides is believed to be mediated by absorptive-mediated endocytosis systems that are based on charge interactions.[8]

Delivery of biopharmaceuticals across the BBB has been reported recently using a related RMT system.[13,14] A carrier protein know as CRM197 was used as safe and effective carrier protein in human vaccines and more recently in anti-cancer trials.[15] CRM197 uses the membrane-bound precursor of heparin-binding epidermal growth factor (HB-EGF) as its transport receptor, which is also known as the diphtheria toxin receptor (DTR). In fact, CRM197 is a non-toxic mutant of diphtheria toxin. Membrane bound HB-EGF is constitutively expressed on various tissues and cells such as blood-brain barrier endothelial cells and several other cells. This means that major sanctuary sites (brain) and cellular reservoirs (T-lymphocytes, monocytes, macrophages) can be reached. Moreover, HB-EGF expression is upregulated strongly under (inflammatory) disease conditions, which will enhance targeted delivery considerably. CRM197 can deliver siRNA across the blood-brain barrier by this mechanism.[16] Other applications may relate to other neurotropic infections (e.g. poliovirus, West Nile virus) or other brain-related diseases (e.g. multiple sclerosis, Parkinson, Alzheimer).

b. Trojan horse liposomes for CNS gene therapy. Gene delivery across the BBB may be ineffective owing to the rapid degradation of extracellular nucleic acids, as well as the pro-inflammatory effects of naked DNA.[17] Encapsulation of plasmid DNA inside pegylated liposomes eliminates the nuclease sensitivity and pro-inflammatory effects of the nucleic acid.[18] Pegylated liposomes, per se, are not transported across the BBB.[19] However, the attachment of a MTH to the tips of the polyethylene glycol strands allows the liposome to engage the BBB RMT system, and this triggers transport of the pegylated immunoliposomes, also called Trojan horse liposomes, across the BBB.[4,20] The administration of this new technology, to mice, rats, or monkeys is followed 24-48 hrs later by global expression of the non-viral transgene in brain.[4,20]

The BBB delivery of immunoliposomes carrying an expression plasmid encoding tyrosine hydroxylase allowed for complete restoration of striatal tyrosine hydroxylase enzyme activity in a model of experimental Parkinson's disease.[21] The intravenous injection of immunoliposomes carrying an expression plasmid encoding a short hairpin RNA directed against the human epidermal growth factor led to a 90% increase in survival time of mice with intra-cranial human brain cancer . The pegylated immunoliposome gene transfer technology enables intravenous RNA interference (RNAi) of the brain.

c. Targeted nanoparticle brain drug delivery systems. Nanoparticles are produced from polymeric precursors, and these structures can be formulated to encapsulate a wide variety of pharmaceuticals.[22] The size of nanoparticles is typically 50-200 nm, and such structures are too large to cross the BBB via free diffusion. However, the formulation of nanoparticles with polysorbate-80 has shown to enable BBB transport.[22] The conjugation of low density lipoprotein (LDL) apoproteins to the surface of nanoparticles appears to trigger RMT across the BBB via the BBB LDL receptor.[23] Recent advances have loaded macrophages with nanoparticle/drug complex ex vivo, followed by the intravenous administration of the cells.[24] Since activated lymphocytes and/or macrophages cross the BBB, these cells may be used as vehicles for drug delivery to the brain.

d. In vivo brain imaging of gene expression. Antisense radiopharmaceuticals hold promise for imaging gene expression in the brain using nuclear medicine imaging modalities, such as PET or SPECT. However, antisense radiopharmaceuticals do not cross the BBB on their own and must be modified if they are to be useful brain gene imaging agents.[25] Peptide nucleic acids can be biotinylated and radiolabeled with 111-indium. In parallel, a conjugate or fusion protein, of avidin and a BBB molecular Trojan horse can by synthesized. The peptide nucleic acid is then coupled to the MTH via the avidin-biotin bridge. Such targeted antisense radiopharmaceuticals cross the BBB, and the brain cell membrane, and enable the in vivo imaging of gene expression in brain.[25]

2. Transporter-independent mechanisms to circumvent the BBB.

a. Intranasal Delivery. A non-invasive, intranasal method of bypassing the blood-brain barrier to deliver therapeutic agents to the brain has been developed.[26,27] This method allows drugs that do not cross the blood-brain barrier to be delivered to the olfactory cerebrospinal fluid via transport across the olfactory region of the nasal epithelium. The surface area of the olfactory region of the nasal epithelium in rodents is large, about 50%, and is small in humans, about 5%,[28] therefore intranasal delivery is not expected to achieve therapeutic drug levels in most brain regions.

b. Convection-enhanced drug delivery (CED). CED is a method for local/regional microinfusion targeted directly to brain tissue. A continuous infusion pressure gradient over hours to days results in distribution of therapeutic agents into the interstitial space. The CED technique is used primarily for large molecular weight agents that show minimal leakage across the BBB and/or have significant systemic toxicity, including viruses, oligonucleotides, nanoparticles, liposome, and targeted immunotoxins.[29] Parameters that affect CED volume of distribution include infusion parameters (rate, volume, duration, cannula size), infusate characteristics (molecular weight, surface properties, tissue affinity), and tissue properties (tissue density, extracellular space, vascularity, and interstitial fluid pressure).[30] Animal studies have demonstrated that the volume of distribution achieved by CED can be imaged by magnetic resonance in real time by including contrast agents within the infusate.[31] The major clinical use of CED will be for targeted therapy of glioblastoma.[29] Recent studies have included interleukin-13/pseudomonas exotoxin alone or in combination with radiation/temozolomide, and radioimmunotherapy with mAbs targeting tenascin or tumor necrosis factor.[32,33] Despite promising early results, it appears that two industry-sponsored phase III trials of CED immunotoxins have been negative. Mechanisms for CED treatment failure include distribution inhomogeneity, high interstitial fluid pressure, and rapid efflux of agent from the injection site.[34] To overcome these issues, increased residence time must be achieved to enhance targeted toxin receptor binding and uptake by the cancerous cells.

Although primarily targeting brain tumors, the CED technique may also gain use for localized neurodegenerative disorders. For example, CED has been used to infuse

glucocerebrosidase into the frontal lobe and brainstem of a patient with neuronopathic Gaucher disease.[35] Infusion of adenovirus vectors or glial-derived neurotrophic factor has been assessed in Parkinson disease.[36]

c. Osmotic BBB Disruption (BBBD). Transient osmotic disruption of the blood-brain, blood-CSF, and blood-tumor barriers can be achieved throughout a vascular circulation by intra-arterial infusion of a hyperosmotic agent, usually mannitol.[37] Osmotic BBBD reversibly opens the BBB by shrinking the cerebrovascular endothelial cells with transient opening of the tight junctions between cells. The BBB is opened to drugs, proteins, and nanoparticles for between 15 minutes (for viral-sized agents) up to 4 hours (for low molecular weight compounds) before returning to baseline permeability.[38] BBBD currently is used clinically for the delivery of chemotherapy to the CNS in patients with brain tumors. BBBD increases parenchymal and CSF chemotherapy concentrations by 10-100 fold compared to intravenous administration alone. Over the past 20 years, 5645 BBBD procedures have been performed in 482 patients by institutions affiliated with the BBB Consortium with minimal adverse side effects. Significant prolongation of survival has been documented in patients with chemoresponsive tumors such as primary CNS lymphoma (PCNSL), without radiotherapy and without cognitive loss.[39] Upcoming studies include use of BBBD to improve delivery of radioimmunotherapeutics in PCNSL and breast cancer metastasis.

Osmotic BBBD also has the potential for enhancing delivery of therapeutics to the brain for treatment of brain infection, lysosomal storage disorders, and neurodegenerative diseases. Studies in the OHSU BBB program have assessed BBBD delivery of antibiotics, enzymes, viral vectors, and nanoparticles in normal rat brain.[40] Delivery of modified siRNA may be a method for targeted gene silencing in the brain. Selective catheterization may be a mechanism to target a local circulation with BBBD. This may be an approach for enhanced delivery of GDNF to the substantia nigra in Parkinson's disease.

d. Bradykinin receptor-mediated BBB opening. Bradykinin, an endogenous peptide mediator of the inflammatory response, can induce transient increases in blood vessel permeability that can be highly specific for tumor vasculature. RMP-7 (lobadimil) is a synthetic

bradykinin analog that is specific for the B2 receptor and is 100-fold more potent than bradykinin in mice. Pharmacological manipulation of the BTB offers the possibility of highly specific opening and targeted drug delivery to tumor, albeit with the possibility that increases in delivery may only be modest and dependent on the tumor type or model treated. Clinical studies in the past 5 years have demonstrated the safety of concurrent RMP-7 and carboplatin, with or without radiation therapy, for both adults and children with gliomas.[41] However, RMP-7 had no effect on the pharmacokinetics or toxicity of carboplatin, and two studies have shown no objective responses of RMP-7 and carboplatin in brain stem glioma or high-grade glioma.[42] Higher doses of RMP-7 may be required to increase carboplatin delivery to tumor, but may also result in increased toxicity in normal brain.

e. Ultrasound-mediated BBB opening. BBB disruption by MRI-guided focused ultrasound can achieve focal CNS delivery in animal models.[43] Consistent vascular leak without tissue damage was achieved by localizing cavitation-generated mechanical stresses to blood vessel walls by IV injection of preformed gas bubbles just prior to pulsed ultrasound treatment. Histology showed that the low power ultrasound caused reversible focal opening which was completely healed within 24 hours.[43] Marker dye extravasation was associated with widening of the tight junctions and active vacuole transport across the endothelial cells. The ultrasound with micro-bubbles exposures did not cause neuronal damage, apoptosis or ischemia, or long term vascular damage. Ultrasound BBB disruption produced clinically relevant levels of liposomal doxorubicin and mAbs in the targeted local areas of the brain in animals. It is as yet unclear whether this technique will show any promise in humans.

3. Imaging in brain drug targeting.

Imaging has made important contributions in drug discovery and brain drug targeting, particularly in areas of pharmacokinetics and in quantifying therapeutic response. Here we focus on recent advances in PET and MRI, but note that seminal contributions have been achieved using optical imaging, radioisotope imaging, and x-ray based techniques as well. It is important to

appreciate the complementary nature of the various imaging techniques, and the potential of multi-modal imaging strategies to greatly accelerate drug discovery.

a. Magnetic resonance imaging. Brain structural imaging data provide important metrics regarding the extent of brain disease and objective surrogate markers for evaluation of therapies. MRI has played an important role in evaluating new CNS therapies, notably in multiple sclerosis,[44,45] stroke,[45] and brain tumor,[46,47] and now is routinely included to provide primary or secondary outcome measures in drug trials of these disease states.[48] MRI provides exceptional soft tissue contrast and sensitivity for focal disease detection which likely will improve with ultra-high field MRI instruments.[49] Quantitative MRI techniques such as relaxography and diffusion based measurements,[50] continue to advance and provide excellent sensitivity for detecting occult disease. These techniques provide a more complete assessment of total brain disease and also will benefit from improved signal to noise associated with higher magnetic field MRI instruments. MRI techniques have been used to track cell migration in the CNS,[51] glioma invasion,[52] and convincing evidence has been presented that increased sensitivity afforded by ultra-high field MRI instruments,[53] may be sufficient to track single cells in vivo.[54]

Increasingly, functional imaging techniques have been used to measure brain physiology in disease and changes associated with treatment. Dynamic susceptibility contrast (DSC) and dynamic contrast enhanced (DCE) MRI studies combined with time-series modeling provide parametric maps of blood volume, blood flow, vascular transit time, vascular permeability, interstitial volume, and water exchange kinetics.[55-58] Recent advances in MRI blood pool contrast agents provide improved measurement of blood volume in the setting of high vascular permeability typically associated with aggressive tumors.[59] These blood pool agents complement traditional low-molecular weight gadolinium agents and are expected to greatly improve MRI measures of blood volume and assessment of new antiangiogenic drugs. Increased research effort in targeted molecular imaging has resulted in important advances. Nuclear medicine techniques have exquisite sensitivity and can be used to measure altered receptor expression associated with disease,[60] and even gene expression.

b. Imaging delivery techniques. The discussion of previous sections makes it clear that multiple strategies exist to circumvent the BBB and achieve therapeutic concentration of small and large molecular weight drugs in the brain. The distribution of drug within the brain using any of the BBB circumvention strategies is complex and imaging information can be used to optimize individual therapies, and importantly, also to accumulate data to improve predictive (*in silico*) models.[61] Contrast enhanced MRI has been used to monitor CED drug delivery and revealed complex distribution patterns resulting from anisotropic diffusion, vascular efflux, and other factors.[62] An important goal of imaging is to investigate the spatial and temporal properties of BBB disruption to understand the potential distribution volume of brain drugs. MRI contrast agents range from the hydrophilic low-molecular weight gadolinium based compounds to large molecular weight iron based nanoparticles. These agents can serve as surrogate markers for drug distribution, and can be used to probe small and large openings in the BBB.[56,63] The continued advancement of MRI techniques for investigation of transient BBB disruption continue to advance, and provide quantitative mapping of transport kinetics and distribution volume.[57]

c. Positron Emission Tomography. PET is non-invasive, has excellent sensitivity and specificity, and has provided quantitative spatially resolved pharmacokinetic and pharmacodynamic measurements on a wide range of small molecule brain drugs in vivo.[60] Advanced PET techniques have been used to determine pharmacokinetics of brain drugs,[60,64,65] pharmacodynamic response following anti-cancer therapy,[66,67] and even in investigations of fetal brain pharmacokinetics following maternal drug administration.[68] PET techniques have the ability to detect very low concentrations of radiolabels, orders of magnitude below pharmacological dose, and are inherently translational. Continued development and application of these techniques for assessing large molecule drugs will facilitate assessment and optimization of brain drug delivery systems.

4. Other important advances that may impact brain drug delivery.

a. BBB genomics. BBB genomics is the application of gene micro-array technologies to the brain microvasculature.[69] The endothelial cells occupy a very small volume of the brain, about

0.1%, or 10^{-3} parts. The sensitivity of gene micro-array is about 10^{-4} parts. Therefore, most BBB-specific transcripts may not be detected in whole brain gene microarray. BBB genomics starts with the isolation of RNA from the brain microvasculature. Subsequently, different technologies, such as suppressive subtractive hybridization,[44] or serial analysis of gene expression,[70] can be employed to identify those genes that are selectively expressed in brain at the brain microvasculature. BBB genomics technologies can lead to new insights into the role the microvasculature plays in brain pathology. Moreover, BBB genomics can also lead to the identification of new BBB transporters, which can then be developed as new conduits to the brain for drug targeting. In parallel with BBB genomics, BBB proteomics programs aim to use protein-base technologies to identify, at the protein level, novel targets within the BBB.[71,72]

b. P-glycoprotein inhibitors. Inhibitors of BBB active efflux transporters, such as P-glycoprotein, have been developed.[73] Such inhibitors may act as co-drugs to increase the brain penetration of P-glycoprotein substrates. This is exemplified in the case of the increased brain penetration of the chemotherapeutic agent, paclitaxel (Taxol®), by co-administration of the P-glycoprotein inhibitor, PSC-833 (valspodar).

c. Conceptualization of the microvascular portion of the blood brain barrier as a component of the neurovascular unit. Increasing experimental evidence indicates that endothelial cell and microvascular properties can be altered by the activation of neurons within the unit that serve the specific vessels.[74,75] Changes in microvascular permeability can affect astrocyte function and neuron integrity. The integration of neuron and microvascular function is a practical framework for considering traffic of agents that might affect or improve neuron function under conditions of injury and inflammation. Neuron function could affect the window for delivery of agents through alterations in endothelial cell-astrocyte communication.

MAJOR UNMET NEEDS IN BRAIN DRUG TARGETING:

1. Need to target therapeutics to specific brain regions or cell types.

It may be possible to engineer a brain-specific large molecule drug using a Trojan horse that only recognizes the endothelium in brain. When the goal is the delivery of the drug to the brain

interstitium, then a single BBB targeting system will be effective. However, when the goal is the delivery of the pharmaceutical to the intracellular space of brain, then the delivery system must be enabled to recognize 2 membranes: the BBB and the brain cell membrane. It is desirable in certain conditions to target a therapeutic to a specific region of brain, e.g. the spinal cord for amyotropic lateral sclerosis or the nigro-striatal tract in Parkinsons. Since drugs that enter the brain via the transvascular system are delivered to all parts of brain, the development of a region-specific targeting system may be difficult for protein drugs. In the case of non-viral gene transfer, regional therapy is possible, owing to the region-specific expression of certain genes in the brain.[4] The use of promoters of these region-specific genes in the engineering of expression plasmids encoding therapeutic genes can enable the selective expression of a transgene to a specific region of the brain. Certain diseases are localized to specific cells in brain, e.g. brain cancer and glial cells, multiple sclerosis and oliogodendrocytes. Once a drug is targeted across the BBB, it may be advantageous to target the drug to a specific cell. This may be possible with the use of bi-specific antibodies, which are engineered to recognize dual targets: the BBB and the specific cell type in brain.

2. **Need to understand toxicity associated with brain drug delivery**. Brain drug targeting with the Trojan horse technologies invariably involves the combination of the neuropharmaceutical with the brain targeting system. Both components of the formulation have the potential for toxicity. Nanomaterials or cellular delivery systems may affect brain capillary endothelial function, including transcytosis and BBB disruption. Thus, it is important to initiate the long term administration of new brain drug targeting systems early in the preclinical research, and to investigate for any untoward cellular effects of these systems. While most toxicity will be detected in the pharmacology and toxicology required by the FDA for an investigational new drug application, or in the phase I clinical trial in small numbers of patients, it is crucial that potential toxic manifestations of the targeting system be evaluated early in the preclinical research.

3. Need to improve understanding of BBB transport systems. There is a lack of molecular information describing the interaction of members of the solute carrier gene family and ATP-binding cassette (ABC) gene family of transporters that participate in the active efflux

transport of drugs and metabolites from brain to blood. Certain members of the ABC gene family, e.g. P-glycoprotein play an important role in the active efflux of drugs across the BBB. However, there are many other members of the ABC gene family, apart from P-glycoprotein, that play a role in efflux across the BBB. In addition, the active efflux of a molecule from brain to blood must involve the coordinated activity of 2 transporters, one localized on the abluminal membrane, and one localized on the luminal membrane. Generally, one transporter, e.g. the ABC transporter, is energy dependent, and the other transporter is energy independent. Candidate energy independent transporters are members of the solute carrier gene family such as the organic anion transporters. The challenge in BBB efflux is to identify the *pairs* of transporters that participate in the active efflux of a given drug. Overall, the database on the modulation of BBB transporters, including CMT, RMT, or efflux systems, is low. In particular, there is a need for expanding the knowledge on how BBB efflux systems are modulated in physiological and pathological conditions. For example, changes in BBB efflux transporters may play a role in drug action in epilepsy.

4. **Need for in vivo evaluation of brain drug pharmacokinetics.** Most therapeutic trial involving drug delivery to the CNS lack basic pharmacology regarding agent delivery.[38] Measurement of brain delivery pharmacokinetics should be a regular component of preclinical, and some clinical studies. Ideally, any new brain drug targeting system should enable the investigator to demonstrate in vivo CNS pharmacological effects following IV administration at reasonable doses of the drug. Methods for quantitative measurements of brain drug uptake remain an issue. Many studies of BBB permeability use the log BB, where BB is the ratio of brain drug concentration to the blood drug concentration at some terminal time point, e.g. 60 min, after administration.[76] The log BB is largely a measure of brain drug volume of distribution, which is determined by cytoplasmic binding of drug to a much greater degree than BBB permeability.[77] A better measure is the percent of injected dose/gram brain.

5. Need to understand of the role(s) of extracellular matrix within the microvascular permeability barrier on component cell function. It is unknown how endothelial cell cohesion and the interaction of endothelial cells with the underlying basal lamina can affect the blood brain

barrier, as well as transport properties of the barrier. Strategies which can facilitate the transport of agents of interest across the microvascular wall could depend for efficacy on the endothelial cell-astrocyte communication, and the contributions of this cross-talk to integrity of the barrier. Indeed, the manner in which these cell components interact to maintain the barrier is still unresolved. How neuron function could also affect the barrier function is understudied. These considerations reinforce the notion that agent delivery depends upon the dynamic nature of the barrier.

6. **Need to identify new brain drug targeting systems.** Multiple combinatorial display systems, incorporating either yeast or phage technology, are presently being mined within the pharmaceutical industry for new drug discovery targets. These combinatorial systems could also be used to screen for new brain drug targeting systems.

7. **Need to speed development and application of molecular imaging probes and targeted contrast agents.** Imaging techniques have the potential to significantly accelerate brain drug development. Targeted molecular probes for MRI and nuclear medicine will improve the specificity of imaging data and aid drug discovery efforts. Imaging agents typically have a much smaller market capitalization than therapeutics, so often are not pursued by the pharmaceutical industry. Many compounds that have unfavorable therapeutical potential could be excellent candidates for imaging probes. Improved access to pharmaceutical data bases could facilitate development of molecular imaging probes. It is important that developed agents be accessible to the research community. Increased industry-academia collaboration in this area could lead to significant synergy, and increased investment in Centers specializing in molecular imaging would be beneficial.

BARRIERS TO FUTURE PROGRESS IN BRAIN DRUG TARGETING:

1. **Lack of training programs for scientists specializing in brain drug targeting.** The development of new brain drug targeting systems requires innovation, and is inherently a high-risk high-reward research area. The disconnect between neuroscience research and drug delivery research makes it difficult to effectively translate progress in the molecular neurosciences

into effective new pharmaceuticals that work in the clinic. There is a need for early integration of brain drug targeting and brain drug discovery in the overall brain drug development mission. However, this is not possible, because there are so few scientists currently being trained in BBB transport biology, in general, much less in brain drug targeting.

2. Lack of NIH funding opportunities targeted to BBB drug delivery research. The BBB and CNS drug delivery is not a major emphasis at the NIH. There is no "BBB transport biology" or "brain drug targeting" in the charter description of multiple study sections at NIH. Brain drug targeting grant applications are generally reviewed not in the context of the targeting technology, per se, but in the context of the drug being delivered for a specific brain disease. Thus, the value of the targeting technology is minimized, and its general applicability is often times not understood or disseminated. This minimization of the BBB and in vivo brain targeting is inherent in a neuroscience enterprise that is so invested in the trans-cranial delivery of experimental therapeutics.

3. Lack of BBB and CNS drug targeting emphasis in the pharmaceutical industry. No large pharmaceutical company has a BBB drug targeting program, which is a total surprise to most lay people. The companies are largely limited to developing the small class of drug that crosses the BBB via lipid-mediation, e.g. the lipid soluble small molecule with a MW<400-500 Da. Development of new potentially curative drugs – recombinant proteins, monoclonal antibodies, antisense, RNAi, gene therapy – appears to lack an understanding of CNS delivery issues. This can result in highly publicized mistakes made whereby costly clinical trials are sponsored on drugs that do not cross the BBB, followed predictably by failure of the phase III clinical trial. The absence of the pharmaceutical industry in the BBB landscape underscores the unique role that the NIH must play in the future expansion of research in this vital area.

4. **Lack of detailed understanding of BBB transport biology**. The BBB is part of the neurovascular unit, which is formed by the capillary endothelium, the pericyte, the astrocyte foot process, and direct neuronal innervation of the microvasculature. The astrocyte foot process is separated from the capillary wall by a short distance occupied by the basement membrane. The paracrine interactions amongst these cells at the brain microvasculature are intense, but poorly

understood. What is needed is a greater understanding of the cellular and molecular biology underlying the maintenance of the neurovascular unit. A derivative of such work would be an expansion of the knowledge of the molecular biology of BBB transport processes within the endothelium. Both ultrastructural, and subcellular fractionation approaches are needed to expand the understanding of BBB transcytosis.

5. Lack of in vivo validation of in vitro BBB studies. There has been an over-reliance on cell culture systems for evaluation of new brain drug targeting systems without appropriate in vivo validation**.** New drugs and brain targeting systems are often primarily evaluated with cell culture models of the BBB in vitro. These in vitro models are deficient in many ways. For example, the BBB permeability coefficient is not replicated in leaky monolayer cultures. The electrical resistance across brain capillaries is estimated to be 8000 Ω cm^2.[78] Conversely, the electrical resistance across the best in vitro BBB models is just 10% of the in vivo value.[79] In vivo the brain uptake of a drug is directly proportional to the plasma concentration-time product of the drug. Many drugs and Trojan horses, especially cationic based targeting systems, may be rapidly absorbed by organs on a single pass, which greatly reduces the plasma concentration-time product of the drug.[80] Such PK considerations are largely eliminated in cell culture. In vitro models do not replicate the neurovascular unit with its multitude of interactions among different cell types that control expression of basement membrane, tight junction, and transporter genes. Certain BBB transporters, e.g., the GLUT1 glucose transporter, or the LAT1 large neutral amino acid transporter, are down-regulated 100-fold in primary culture of brain capillary endothelium.[81,82] If in vitro models of the BBB are to be used, there should be attempts to make in vivo/in vitro correlations to validate the in vitro model.

8. Lack of controlled clinical trials addressing CNS drug delivery. The major goal of any new brain drug targeting technology is the translation of the technology from laboratory science to clinical medicine. This has proven quite difficult, for any brain delivery system. Most examples of the translation of a brain drug delivery system into the clinic involve non-targeted approaches, e.g. osmotic BBBD, biochemical BBB modification such as with bradykinin analogues, or trans-cranial delivery systems, e.g. intracerebroventricular administration, or

convection enhanced diffusion. Such clinical trials have been largely single institution phase II trials rather than controlled phase III studies. What is needed is the translation of brain targeting methodologies, including targeted delivery systems that are developed to the extent that clinical trials can begin. Despite the difficulty in translating new targeting technologies to the clinic, the recent applications of genetic engineering and the construction of fusion genes which allow for manufacturing of fusion proteins,[7] offer the promise that engineered protein drugs fused to molecular Trojan horses will enter clinical trials.

IMPORTANT FUTURE RESEARCH AREAS IN BRAIN DRUG TARGETING:

An overall goal of future research in brain drug targeting is to expand the CNS drug space from lipid-soluble small molecules to the much larger space of pharmaceutics that include molecules that do not normally cross the BBB. The following specific areas have been identified:

1. Identify new BBB transporters that could be portals of entry for brain drug targeting systems.
2. Develop brain drug targeting systems that enable the brain delivery of recombinant protein neurotherapeutics.
3. Validate new drug targeting systems using in vivo models.
4. Optimize pharmacokinetics of in vivo brain drug targeting systems.
5. Develop genomic and proteomic discovery platforms that enable the identification of new BBB transporters.
6. Apply protein-based therapeutics in specific brain diseases, including stroke, neurogenesis, e.g., as an adjuvant to stem cell therapy.Greater understanding of the function of active efflux transporters at the BBB.
7. Improve understanding of the regulation of BBB transport by astrocyte foot processes.

8. Improve understanding of the interaction of the neuronal and microvascular (endothelial cell-astrocyte endfoot) components of the neurovascular unit, their participation in the permeability barrier, in transport receptor expression, and their facilitation of the passage of agents into the neuropil.

Many of the drug discovery steps listed above require the application of advanced in vivo imaging techniques to facilitate both preclinical and clinical investigations. Neuropharmacokinetic and neuropharmacodynamic measurements with good spatial and temporal resolution will be important in the evaluation of various brain drug delivery systems. The functionality of brain barrier systems is known to change under various pathological conditions, and these changes need to be characterized. For example, the in vivo spatial distribution of various RMT systems in health and disease is important to investigate if drug deliver is to be optimized. Multiple imaging modalities are likely to be necessary and are expected to provide important information at different phases of the brain drug discovery process. Therefore, an important goal associated with brain drug targeting is an increased emphasis on development of novel imaging agents and techniques for optical, nuclear, x-ray, and magnetic resonance imaging.

Finally, to facilitate the specific goals listed above we recommend the creation of cross-disciplinary, integrated centers that bring together transport biologists, pharmaceutical scientists, bioengineers, and imaging scientists focused on the development of new brain drug targeting systems.

CONSENSUS FUTURE PRIMARY PRIORITY IN BRAIN DRUG TARGETING:

Group consensus on the primary priority in future brain drug targeting converged on the need for the establishment of 'BBB Drug Targeting Centers,' which are cross-disciplinary, integrated centers that bring together transport biologists, pharmaceutical scientists, and bioengineers that can develop new brain drug targeting systems of the future. Such centers

would develop technology platforms for both small molecule and large molecule pharmaceuticals. The center would be technology-based, and applied science-based, and focused on the development of practical delivery technologies that could be used to re-formulate drugs that normally do not cross the BBB. The emphasis on technology would make the center a model of translational research within the neurosciences. However, the center should also be grounded in the basic science of the brain capillary endothelium, and brain microvascular transport biology, thus illustrating the continuum between the basic and applied sciences of brain drug targeting research.

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Figure Legends

Figure 1. Patterns of blood-brain barrier disruption in gliobastoma multiforme (GBM). The T1-weighted 3T MRI in panels a-d were acquired from a 49 y F. a) pre-contrast, b) 20 minutes post 0.1 mmol/kg gadoteridol, c) 20 minutes post 1 mg/kg Ferumoxytol, and d) 24 hours post Ferumoxytol administration. The low-molecular weight gadolinium (Gd) shows widespread distribution in the tumor region (panel b). The large molecular weight iron (Fe) compound shows no significant extravasation at 20 minutes post administration (panel c), but does extravasate by 24 hours post administration (panel d), albeit to a lesser extent than gadoteridol (panel b). Rapid extravasation of the low-molecular weight Gd agent confounds measurement of blood volume in dynamic MRI studies. The problem is obviated with blood pool contrast agents, such as Ferumoxytol, are used. Modeling the dynamic contrast enhancement provides measurement of BBB permeability and local tissue concentration for the different size contrast agents. This information can be used to estimate vascular changes associated with therapy, and also to estimate the passive diffusion from plasma into brain parenchyma of small and large drugs.

Figure 2. Comparison of dynamic susceptibility contrast (DSC) temporal plots for low and high-molecular weight contrast reagents (CR). Signal intensity plots from a region of interest (ROI) in a contrast enhancing tumor region (see circular ROI in inset image) for low molecular weight (gadoteridol; Gd, blue diamonds) and high-molecular weight (Ferumoxytol; Fe, red squares) reagents are shown. The Fe administration resulted in a 15% drop in signal intensity ~27 s after intravascular injection. A pseudo-steady state signal level was realized by about 50 s after Fe administration. The Gd administration also resulted in a signal minimum ~27 s after injection. However, extravasation of the Gd compound results in a continued increase in signal intensity over the dynamic measurement, and systematic error in DSC modeling. The much large Fe agent does leak into the brain (see Figure 1d), but with a much smaller rate constant that the Gd compound.

Figure 3. Cerebral blood volume (CBV) and mean transit time (MTT) maps from DSC modeling of a Ferumoxytol bolus injection in an individual with GBM. Data were acquired using a 3T MRI instrument from the same subject of Figures 1 and 2. A relative color scale for the two measures is displayed at the right. Reduced CBV and prolonged MTT are evident in the peri-tumor regions in the right hemisphere.

Figure 1

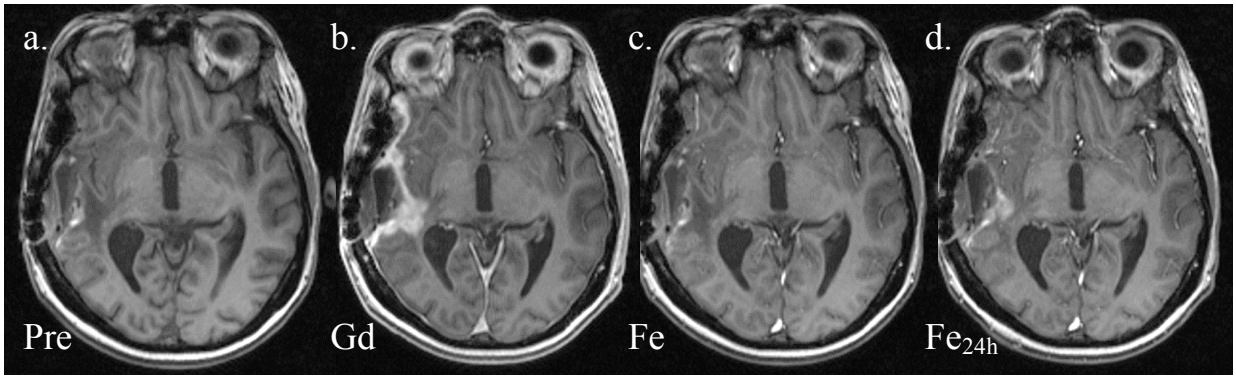

Figure 2

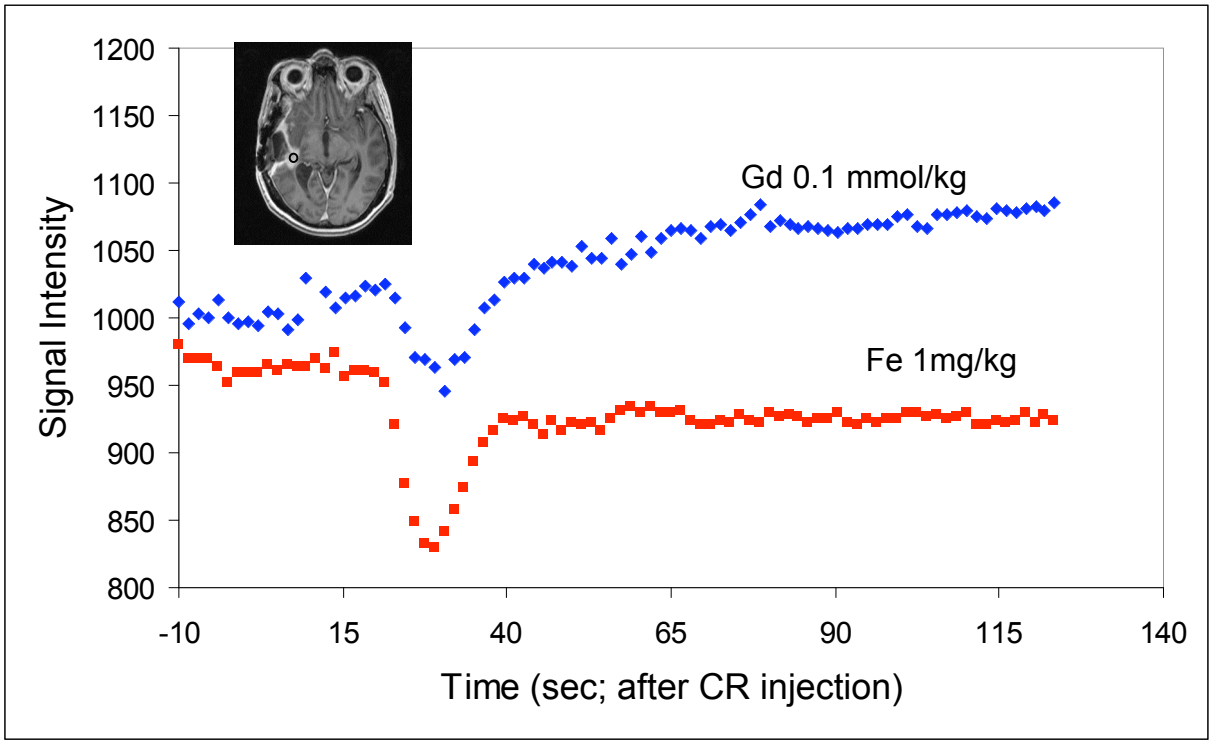
1200
1150
1100
1050
1000
950
900
850
800
Signal Intensity
Gd 0.1 mmol/kg
Fe 1mg/kg
-10
15
40
65
90
115
140
Time (sec; after CR injection)

Figure 3