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Blood–brain barrier delivery

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Neuropharmaceutics is the largest potential growth sector of the pharmaceutical industry. However, this growth is blocked by the problem of the blood–brain barrier (BBB). Essentially 100% of large-molecule drugs and >98% of small-molecule drugs do not cross the BBB. The BBB can be traversed because there are multiple endogenous transporters within this barrier. Therefore, brain drug development programs of the future need to be re-configured so that drugs are formulated to enable transport into the brain via endogenous BBB transporters.

Introduction

The most important factor limiting the development of new drugs for the central nervous system (CNS) is the blood–brain barrier (BBB). The BBB limits the brain penetration of most CNS drug candidates. The BBB phenomenon is illustrated by Figure 1a. Radiolabeled histamine, a small molecule of just 111 Da, was injected intravenously into an adult mouse, and the animal was killed 30 mins later for whole-body autoradiography [1]. The study shows that the small molecule readily penetrates into the post-vascular space of all organs of the body, except for the brain and spinal cord. The limited penetration of drugs into the brain is the rule, not the exception. Essentially, 100% of large-molecule pharmaceutics, including peptides, recombinant proteins, monoclonal antibodies, RNA interference (RNAi)-based drugs and gene therapies, do not cross the BBB [2]. A common misconception is that small molecules readily cross the BBB. However, in fact, >98% of all small molecules do not cross the BBB either. There are >7000 drugs in the Comprehensive Medicinal Chemistry (CMC) database, and only 5% of these drugs treat the CNS, and the drugs that do treat the CNS are limited to treatment of just three conditions: depression, schizophrenia and insomnia [3]. In another study, 12% of all drugs are active in the CNS, but only 1% of all drugs are active in the brain for diseases other than affective disorders [4].

The fact that so few drugs cross the BBB becomes particularly problematic considering that the number of individuals with a CNS condition will grow with an aging population. The number of people older than 65 years will increase by 50% by 2020, and the annual US expenditures for Alzheimer's Disease (AD) alone could approximate US$0.5 trillion at that time. The neuropharmaceutical market should be the largest sector in the industry because one from every three individuals will have a CNS condition during their lifetime [5]. Yet, the global CNS pharmaceutical market would have to grow >500% just to equal the cardiovascular market [6]. Considering the potential size of the global CNS pharmaceutical market, and considering that so few drugs cross the BBB, one would expect that the development of BBB drug delivery technologies would be a high priority in the pharmaceutical industry and in the academic sciences. In fact, there is not a single medium or large pharmaceutical company in the world today that has a BBB-drug-targeting technology program. Even if big pharma wanted to change this situation, there would be no staff to hire because there are so few BBB scientists being trained in academia. In the USA, there is not a single academic neuroscience program that has any emphasis on BBB drug targeting technology. One routinely reads summaries of workshops in either the USA or Europe that are devoted to various CNS diseases such as AD, Parkinson's disease (PD), brain cancer or stroke, and the issue of BBB drug delivery is not even mentioned.

The BBB drug delivery problem can be solved, but this requires new approaches to this area of pharmaceutics. The old ways of drilling a hole in the head for trans-cranial brain drug delivery, or medicinal chemistry attempting to lipidize a water-soluble small molecule, must give way to new approaches. The new technology is based on knowledge of endogenous BBB transporters, and aims to reformulate drug structures so that these molecules can cross the

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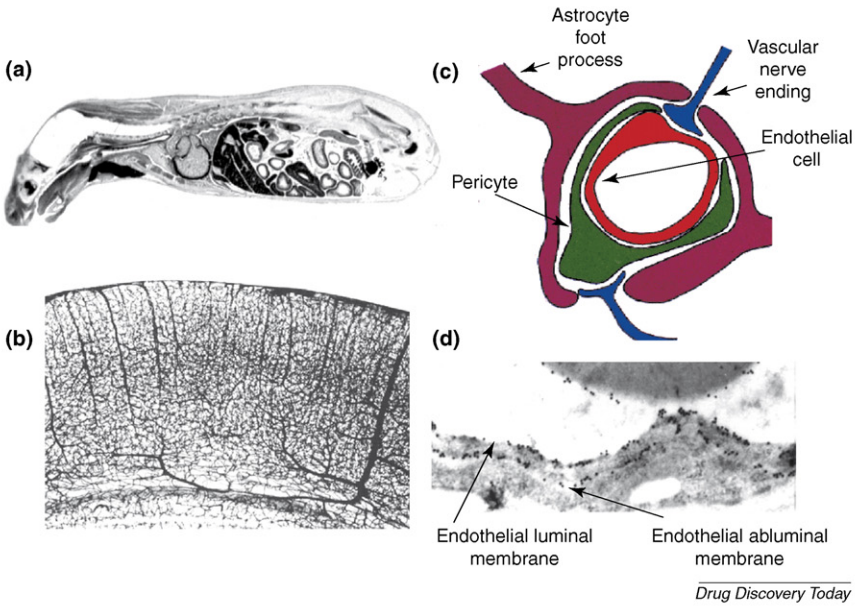

FIGURE 1

The blood–brain barrier. (a) Whole-body autoradiogram of a mouse administered radiolabeled histamine intravenously [1]. The small molecule (i.e. 111 Da) enters all organs of the body, except for the brain and spinal cord. Histamine does not enter the CNS because histamine does not cross the blood–brain barrier (BBB). **(b)** India-ink study of the rat brain [52] shows the complexity of the cerebral microvasculature, which is the site of the BBB. There are >100 billion capillaries in the human brain and every neuron is virtually perfused by its own blood vessel. **(c)** The brain microvasculature comprises four cells: the endothelium, the pericyte, the astrocyte foot process and the capillary nerve ending [78]. However, the permeability properties, *per se*, of the BBB are controlled by the capillary endothelial cell. **(d)** Immunogold electron microscopic study of the human brain, with an antibody to the GLUT1 glucose transporter, shows the expression of this endogenous BBB transporter on the luminal and abluminal membranes of the brain capillary endothelial cell [54].

BBB via endogenous transport systems. This is a radical departure from existing practices in CNS drug development. However, unless changes are made, the future of CNS drugs will be limited to the small class of drugs that cross the BBB via lipid-mediated free diffusion: lipid-soluble small molecules with a molecular weight (MW) <400 Da. These drugs treat only a handful of CNS conditions, generally restricted to affective disorders, epilepsy and insomnia [7].

The importance of new approaches to brain drug development is illustrated by considering the limitations of the existing brain drug delivery strategies. These delivery systems include trans-cranial brain drug delivery, trans-nasal brain drug delivery, BBB disruption and small molecule lipidization.

Trans-cranial drug delivery to the brain

Drugs can be delivered to the brain by first drilling a hole in the head, and this encompasses three basic delivery methods: intra-cerebroventricular (ICV) injection, intra-cerebral (IC) implantation and convection-enhanced diffusion (CED). The ICV administration of glial-derived neurotrophic factor (GDNF) was recently attempted for the treatment of PD [8]. There was no therapeutic effect in patients because the neurotrophic factor did not reach the striatum of brain, and there was a significant number of adverse events related to the trans-cranial delivery system. In fact, the penetration of a neurotrophin such as GDNF into the caudate-putamen nucleus of brain parenchyma from the cerebrospinal fluid (CSF) flow tracks is not expected. The rate of CSF exodus from brain is much faster than the rate of solute diffusion into brain parenchyma from the ependymal surface [2]. Diffusion is a slow process compared to the rapid bulk flow of CSF in brain. The 140 ml volume of CSF in the human brain is completely turned over every 4–5 h, and exits the brain to blood. Moreover, ICV drug delivery to the brain results in high drug exposure at the ependymal surface of the brain, which can cause a subependymal astrogliotic reaction [9,10]. A paradox of ICV drug administration is that the drug distributes to the blood much better than it does to the brain [11], and an ICV injection is similar to a slow intravenous (IV) injection [12]. Drugs or genes have been injected into the brain by the IC implantation route. However, drug diffusion decreases exponentially with the diffusion distance. Accordingly, the concentration of drug is reduced 90% at just 500 microns removed from the drug injection site following IC implantation [13]. In the case of CED, convection replaces diffusion as fluid is continuously infused into the brain via a catheter at a fixed flow rate. However, the brain and spinal cord are the only organs in the body that lack a lymphatic system and the CNS lacks mechanisms to efficiently clear exogenous fluid. Consequently, the fluid that is infused into the brain moves preferentially along white matter tracks [14,15]. The pathway of fluid movement in the brain with CED can be traced by staining the brain with an antibody to glial fibrillary acidic protein (GFAP) [15], which is a marker of astrogliosis. Astrogliosis can be a precursor to demyelination, which is observed in the primate brain around the CED catheter [15].

Trans-nasal drug delivery to the brain

The nasal instillation of lipid-soluble small molecules, such as progesterone, results in a CSF concentration of drug that exceeds

the plasma concentration [16], which indicates a direct movement of the drug from the submucus space of the nose into the CSF compartment of brain. Following diffusion across the nasal mucosal barrier, the drug can cross the arachnoid membrane and enter into olfactory CSF. Once there, the drug will move along the usual CSF flow tracks. Therefore, delivery of drug to the brain via the nose is very similar to an ICV injection. Lipid-soluble small molecules move from the nose to the olfactory CSF to the general CSF, and then to the blood via CSF absorption at the superior sagittal sinus. However, most CNS drug candidates are not the lipid-soluble small molecules that freely cross membrane barriers, including the nasal mucosa. Most pharmaceuticals are water soluble or have MWs >400 Da, and do not freely traverse biological barriers. Nevertheless, certain drugs that would not be expected to cross the nasal mucosa have been found to enter into olfactory CSF following intra-nasal instillation. This is because of the fact that local injury has been induced with the trans-nasal delivery system, thus leading to a breakdown of the nasal mucosal barrier. In the human nose, a volume >100 μl per nares will result in local nasal injury [17]. What one finds in many rodent studies is the forced nasal administration of large volumes, such as 50 μl per rat nares, which causes local injury and breakdown of the normal nasal barriers. For drugs other than lipid-soluble small molecules, local injury to the nasal barriers could be a prerequisite for drug transport between the nasal and olfactory CSF compartments [18].

Once instilled into the nasal cavity, drug must cross the olfactory part of the nasal epithelium to enter into the submucus space adjacent to the olfactory CSF. Whereas the fraction of the nasal mucosa that is olfactory in the rat is 50% of the total surface area of the nose, in humans, the olfactory part of the nasal mucosa is only 3–8% [19–23]. One would predict that when drug is administered via trans-nasal delivery in a volume that does not cause local injury, the drug will not distribute to the CSF, and this is what has been experimentally observed for small molecules such as melatonin or vitamin B12 [17,21].

BBB disruption

The BBB can be transiently disrupted by a variety of means such as intra-carotid arterial infusion of hyperosmotic solutions, noxious agents including vasoactive compounds or local ultrasonic irradiation of the brain. The problem with BBB disruption is that this approach to brain drug delivery allows for the leakage of plasma proteins into the brain. Albumin is toxic to astrocytes [24], and astrogliosis is induced when brain comes in contact with blood. BBB disruption leads to vascular pathology [25] and to chronic neuropathologic changes in the brain [26]. Although BBB disruption is unlikely to be widely used as a brain drug delivery strategy, it is surprising the extent to which the BBB is inadvertently disrupted during the course of new drug testing. This leads to the presumption that a drug candidate, which does not cross the BBB, is a brain-penetrating molecule. Subsequently, once the drug enters clinical trials, the BBB disruption causes toxicity. Inadvertent BBB disruption (IBD) is typically caused by the presence of a noxious molecule in the drug formulation, such as adjuvants, solvents or stabilizers.

Inadvertent BBB disruption with CNS vaccines

The active immunization against target antigens has been proposed for several CNS conditions [27], including AD [28]. The goal is to immunize the patient with AD against the amyloid-β (Aβ) peptide that forms brain amyloid. AD-transgenic mice were immunized with Aβ and complete Freund's adjuvant (CFA) [28]. The mice developed high titers of anti-Aβ antibodies in blood. However, such antibodies must cross the BBB to clear the brain of Aβ amyloid. Clearance of brain amyloid was observed in the mice, which indicated that the antibodies were able to enter brain [28]. Because most antibodies do not cross the BBB, the clearance of brain amyloid suggested the BBB became disrupted during the course of the immunization. The adjuvant of CFA includes mannan, a component of the *Mycobacterium tuberculosis* cell wall, and the intravenous administration of anti-mannan antibodies results in immediate BBB disruption [29]. Moreover, the administration of CFA to mice is known to increase BBB permeability [30], presumably through the induction of anti-mannan antibodies. Subsequently, human clinical trials were aborted in phase II as patients developed an encephalitis-like clinical picture following Aβ active immunization [31].

Inadvertent BBB disruption with solvents and stabilizers

Solvents such as sodium dodecyl sulfate (SDS), ethanol, dimethylsulfoxide (DMSO), glycerol and polysorbate-80 (Tween-80) are frequently included in drug formulations, and are administered to animals in doses that cause BBB disruption. Low systemic doses of SDS cause BBB disruption [32], and the CNS effects of a cytokine have been subsequently traced to the SDS in the formulation [33]. Doses of glycerol, ethanol or DMSO >1 g/kg cause solvent-mediated BBB disruption [34,35]. The administration of 0.2 ml of a 50% solution of either ethanol or DMSO to a 25 g mouse is equivalent to a dose of 4 g/kg. Polysorbate-80 is a popular stabilizer in a variety of drug formulations, but low doses, 3 mg/kg, of this detergent cause BBB disruption [36]. CNS effects of systemic administration of a neuropeptide, which does not cross the BBB, could only be elicited if the neuropeptide was co-administered with polysorbate-80 [37].

Lipidization of small molecules

There is a significant effort in the pharmaceutical industry to use medicinal chemistry to convert water-soluble drugs that do not penetrate the BBB into lipid-soluble drugs that do cross the BBB. Alternatively, lipid carriers are attached to water-soluble drugs [38]. However, in actual practice, the reformulation of a water-soluble drug with lipidization modifications is difficult to execute successfully, and there is not a single example of a drug presently sold whereby medicinal chemistry was successfully used to convert a non-brain-penetrating drug into a molecule that crosses the BBB in pharmacologically significant amounts. The one exception to this is the acetylation of morphine to form heroin [39]. Morphine has two hydroxyl groups and acetylation of both hydroxyl groups results in the formation of heroin. The removal of each hydroxyl group results in the removal of two hydrogen bonds formed between the drug and solvent water. As a general rule, the BBB permeability of a drug decreases 1 log order in magnitude for each pair of H-bonds added to the molecule in the form of polar functional groups [40]. Based on H-bonding rules [41,42], the number of H-bonds that a given drug forms with water can be calculated by inspection of the chemical structure. Once the number of H-bonds is greater than eight, it is unlikely that the drug

BOX 1

Two-step method for prediction of whether a small-molecule drug crosses the BBB via lipid-mediated free diffusion

Step 1: determine molecular weight (MW) of drug
Step 2: determine H-bonding based on drug chemical structure
H-bonding rules:
4 H-bonds for each terminal amide group
3 H-bonds for each internal amide, primary amino group or carboxyl group
2 H-bonds for each hydroxyl group
1 H-bond for each ether or carbonyl group
Add total H-bonds formed between drug and solvent water

Parameter	Unrestricted BBB transport	Restricted BBB transport
MW	**<400 Da**	**>400 Da**
Total H-bonding	**<8**	**>8**

If the MW of the drug is >400 Da and/or the drug forms eight or more H-bonds, then the drug is probably a poor CNS-penetrating molecule.

Certain functional groups abort BBB transport, for example:
Quaternary ammonium group
More than one carboxyl group

crosses the BBB via lipid-mediated free diffusion in pharmacologically significant amounts (Box 1).

The other important parameter determining free diffusion of small molecules across the BBB is MW. Once the MW is >400 Da the BBB permeability of the drug does not increase in proportion to lipid solubility [43]. The MW threshold effect of solute-free diffusion across biological barriers has been known for many years [44–46]. The biophysical basis for the MW threshold appears to be the transitory formation of pores within the phospholipid bilayer that are created as the free fatty acyl side-chains kink in the process of normal molecular motion within the phospholipid bilayer [47,48]. The pores are of finite size and restrict the movement of small molecules that have a spherical volume in excess of the pore volume. BBB permeation decreases 100-fold as the surface area of the drug is increased from 52 A^2 (e.g. a drug with a MW of 200 Da), to 105 A^2 (e.g. a drug with a MW of 450 Da) [43]. Based on the MW and H-bonding for a given drug, a reasonable prediction can be made as to whether the drug crosses the BBB in pharmacologically significant amounts via lipid-mediated free diffusion (Box 1).

Apart from H-bonding and MW, a third important factor determining brain availability of a pharmaceutical is the plasma pharmacokinetics and the plasma area under the concentration curve (AUC). The concentration of drug in brain is directly related to (i) the BBB permeability coefficient (Pe), and (ii) the plasma AUC [2]. Lipidization of the molecule can increase the BBB Pe; however, lipidization of a molecule will increase the uptake in all organs of the body, resulting in a decrease in plasma AUC [49]. The brain uptake of the drug, expressed as percentage of injected dose (ID/g), decreases in proportion to the decrease in plasma AUC caused by lipidization of the drug. Thus, lipidization increases the Pe but decreases the plasma AUC, and these factors can have offsetting effects, resulting in little change in the brain %ID/g.

The problems associated with lipid-mediated transport of solute across the BBB can be eliminated by redirecting medicinal chemistry efforts away from increasing lipid-mediated transport of the drug and toward increasing carrier-mediated transport of the drug. Medicinal chemistry can be used to modify the drug to make it a substrate for various endogenous carrier-mediated transporters (CMT) at the BBB. For example, dopamine does not cross the BBB. However, the conversion of this catecholamine to an α-amino acid, L-dopa, results in a CNS active drug. The L-dopa crosses the BBB by CMT [50], via a system now known to be the large neutral amino acid type 1 transporter (LAT1) [51]. Once inside brain, L-dopa is decarboxylated by aromatic amino acid decarboxylase to generate the original dopamine pharmaceutical. This model could be replicated for numerous other drugs. However, the use of medicinal chemistry to optimize chemical structure to enable transport via endogenous BBB transport pathways requires a knowledge-base of the endogenous BBB transporters.

Endogenous BBB transporters

The anatomical basis of the BBB is the brain microvascular endothelial barrier. An India-ink study of the rat brain (Figure 1b) shows the enormous complexity of the cerebral microvasculature [52]. There are >100 billion capillaries in the human brain and each neuron is virtually perfused by its own blood vessel [53]. The length of capillaries in the human brain is ~400 miles and the surface area of the BBB in the human brain is ~20 m^2 [2]. The brain microvasculature comprises four cells, which are depicted in Figure 1c. The microvascular cells of the brain include the endothelial cell, the pericyte, which shares the basement membrane with the endothelial cell, the astrocyte foot process, which invests ~90–98% of the brain surface of the microvasculature, and nerve endings that end directly on the vascular surface. Although all four cells contribute to the functioning of the microvasculature in brain, the permeability properties, *per se*, of the BBB are controlled only by the capillary endothelial cell. The astrocyte, the pericyte and the basement membrane do not constitute any significant permeability barrier to solute exchange between blood and brain interstitial fluid. Movement of solute across the capillary endothelial barrier is a process of movement through two membranes in series, the luminal and abluminal membranes of the capillary endothelial cell (Figure 1d). These two membranes are separated by only 200 nm of endothelial cytoplasm. The endogenous transporters of the BBB are expressed on the luminal and abluminal membranes of the brain capillary endothelial cell. The expression of the GLUT1 glucose transporter on both of these membranes of the human BBB is shown by the electron microscopic immunogold study in Figure 1d [54]. The use of immunogold electron microscopy is the most direct method for localization of BBB transporters to the luminal versus abluminal membranes of the brain capillary endothelium [54].

The endogenous BBB transporters can be classified into three categories: CMT, active efflux transport (AET) and receptor-mediated transport (RMT). Whereas the CMT and AET systems are responsible for the transport of small molecules between blood and brain [55,56], the RMT systems are responsible for the transport across the BBB of certain endogenous large molecules. Examples of CMT, AET and RMT systems that are expressed at the BBB are listed in Figure 2. The CMT systems usually mediate brain to blood influx

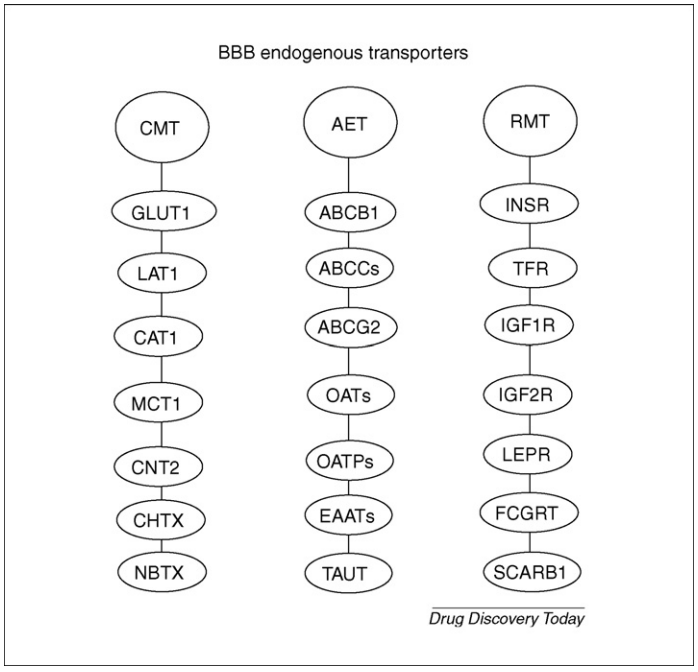

FIGURE 2

Endogenous blood–brain barrier transporters. The transporters are grouped into three categories: carrier-mediated transport (CMT), active efflux transport (AET) and receptor-mediated transport (RMT).Abbreviations: GLUT1, glucose transporter, member 1 (SLC2); LAT1, large neutral amino acid transporter, member 1 (SLC7); CAT1, cationic amino acid transporter, member 1(SLC7); MCT1, monocarboxylic acid transporter, member 1 (SLC16); CNT2, concentrative nucleoside transporter, member 2 (SLC28); CHT, choline transporter (SLC5); NBT, nucleobase transporter; ABCB1, adenosine triphposphate-binding cassette (ABC) transporter, subfamily B, member 1, also called P-glycoprotein; ABCC, ABC transporter, subfamily C; ABCG2, ABC transporter, subfamily G, member 2; OAT, organic anion transporter (SLC22); OATP, organic anion-transporting polypeptide (SLC21); EAAT, glutamic acid amino acid transporter (SLC1); TAUT, taurine transporter (SLC6); INSR, insulin receptor; TFR, transferrin receptor; IGFR, insulin-like growth factor receptor; LEPR, leptin receptor; FCGRT, Fc fragment of IgG receptor transporter, also called neonatal Fc receptor (FCRN); SCARB1, scavenger receptor, class B, member 1. The X designation for the CHT and NBT transporter indicates these transporters have not yet been cloned or identified at the molecular level for the blood–brain barrier (BBB).

of substrate, although the CMT systems can also mediate brain to blood efflux. The AET systems usually mediate brain to blood efflux of substrate. The classification of certain transporters as CMT or AET systems (Figure 2) is provisional, pending further investigations. Certain transporters classified as AET systems in Figure 2, for example OATs or OATPs, can function as CMT systems.

Carrier-mediated transport

The GLUT1 glucose transporter transports glucose and other hexoses, including 2-deoxyglucose and fluoro-deoxyglucose used in positron emission tomography (PET) scanning. LAT1 transports large and small neutral amino acids, as well as certain amino acid drugs including L-dopa, α-methyl-dopa, α-methyl-*para*-tyrosine or gabapentin. Gabapentin is a γ-amino acid that (owing to its cyclic structure) is recognized by the LAT1 transporter, and which transports only α-amino acids. The cationic amino acid transporter CAT1 transports basic amino acids, such as arginine or lysine. The monocarboxylic acid transporter MCT1 transports lactate, pyruvate, ketone bodies and certain monocarboxylic acid drugs such as probenecid. The concentrative nucleoside transporter CNT2 transports purine nucleosides, and certain pyrimidine nucleosides such as uridine. The purine bases, but not the pyrimidine bases, undergo carrier-mediated transport across the BBB [57]. However, the nucleobase transporter (NBT) that is expressed at the BBB has yet to be cloned. Choline undergoes carrier-mediated transport across the BBB via a sodium-independent process [58]. Although sodium-dependent choline transporters (CHT) have been cloned, the sodium-independent CHT at the BBB has not been cloned. The various CMT systems provide a diverse space of molecular structure that could be mimicked with medicinal chemistry modifications of drugs that normally do not cross the BBB. The difficulty would be in maintaining activity of the drug for its cognate receptor in brain beyond the BBB. Alternatively, certain medicinal chemistry strategies could be employed that render the molecule susceptible to enzymes in the brain that convert the modified drug back to the parent drug once it crosses the BBB. Although the BBB CMT systems are saturable at high substrate concentration, the transporters are not effectively saturated by endogenous drug *in vivo*. This is because the K_m of the BBB CMT systems is >10-fold above the existing plasma concentration of drug.

Active efflux transport

The most studied AET system at the BBB is P-glycoprotein, which is a product of the ABCB1 gene (Figure 2). However, BBB AET transport biology extends beyond P-glycoprotein. There are multiple other members of the ABC gene family that represent energy-dependent active efflux transporters at the BBB, including members of the ABCC and the ABCG2 gene family (Figure 2). In addition, active efflux transport of drug from brain to blood is a process mediated by two transporters in series, including an energy-dependent transporter and an energy-independent transporter [7]. The energy-dependent transporter from the ABC gene family can be expressed at the luminal membrane, whereas the energy-independent transporter can be expressed at the abluminal membrane of the brain capillary endothelial cell. The energy-independent transporters are members of the solute carrier (SLC) gene families and include members of the OAT or OATP organic anion transporters (Figure 2), acidic amino acid transporters such as members of the EAAT family or active efflux transporters such as the TAUT taurine transporter (Figure 2). It is also possible that the energy- or sodium-dependent transporter is present at the abluminal membrane, whereas the energy-independent transporter is present at the luminal membrane. The exact location of the transporters within the endothelial membrane is best-determined with electron microscopy, such as shown in Figure 1d for the GLUT1 glucose transporter [54]. However, there have been few electron microscopic localizations of AET transporters at the luminal and abluminal membranes of the brain capillary endothelial cell. The challenge in understanding AETs at the BBB is to identify the pair of energy-dependent and energy-independent transporters that work in concert to mediate the active efflux of a given pharmaceutical, and to localize these transporters to the respective luminal or abluminal endothelial membrane.

Receptor-mediated transport

Certain large-molecule peptides in the blood undergo RMT across the BBB via the endogenous peptide receptors (Figure 2) [2].

Insulin in blood undergoes RMT across the BBB via the endogenous BBB insulin receptor (INSR). Brain iron is derived from circulating transferrin via RMT across the BBB transferrin receptor (TFR). The insulin-like growth factors (IGF) IGF-1 and IGF-2 undergo RMT across the BBB via a separate type 1 and type 2 IGF receptors (IGF1R and IGF2R) in rodents. The IGF2R also transports proteins conjugated with mannose-6-phosphate. However, the IGF receptor at the human BBB differs from the IGFR at the animal BBB, in that both IGF-1 and IGF-2 bind with high affinity to a single variant IGFR [59]. Unlike insulin or transferrin, the circulating IGFs are >99.9% bound by IGF-binding proteins. The BBB expresses the short form of the leptin receptor (LEPR) [60], and this receptor might participate in the RMT of leptin across the BBB *in vivo*, although definitive evidence for this has yet to be reported. IgG present in brain is rapidly exported to blood via reverse transcytosis on the BBB neonatal Fc receptor [61,62], also called FcRn or FCGRT (Figure 2). This is an asymmetric RMT system because the BBB FcRn does not mediate the transport of IgG from blood to brain. Modified lipoproteins, such as acetylated low density lipoprotein (LDL), undergo receptor-mediated endocytosis into the brain capillary endothelial cell via the scavenger receptor type B1 (SCARB1). However, this is not a transcytosis system because acetylated LDL in blood is completely sequestered by the brain capillary endothelium, and does not transcytose through the endothelium to enter the brain interstitial fluid from blood [2].

BBB transport of large-molecule drugs with molecular Trojan horses

Certain peptidomimetic mAbs undergo RMT across the BBB *in vivo* [2]. The receptor-specific mAb binds to an exofacial epitope on the endogenous BBB peptide receptor, at a site that is spatially removed from the endogenous ligand binding site, and 'piggybacks' across the BBB on the endogenous peptide RMT system. The most potent BBB molecular Trojan horse known to date is a mAb for the human insulin receptor (HIR), which is active at both the BBB of humans and the BBB of Old World primates such as Rhesus monkeys [2]. The most active molecular Trojan horses in rats and mice are the murine OX26 mAb for the rat TFR and the rat 8D3 mAb for the mouse TFR, respectively. These receptor-specific mAbs are species-specific. The OX26 mAb is not active in mice and the 8D3 mAb is not active in rats. The HIRmAb is not active in rodents or in New World primates such as squirrel monkey [2]. Therefore, in the case where the molecular Trojan horse is a receptor-specific mAb, the molecular Trojan horse that is used in preclinical research will be different from the molecular Trojan horse developed for human therapeutics.

Peptides, recombinant proteins and antisense agents such as peptide nucleic acids (PNA) have been conjugated to rat or mouse molecular Trojan horses for delivery across the BBB *in vivo* following intravenous administration. The molecular Trojan horse carries the large molecule pharmaceutical across the BBB *in vivo*, to cause *in vivo* CNS pharmacological actions. This has been reduced to pharmacologic practice *in vivo* for multiple drugs and experimental model systems (Table 1). Vasoactive intestinal peptide (VIP) is a potent cerebral vasodilator when applied topically to brain vessels. Intra-carotid VIP does not increase cerebral blood flow because VIP does not cross the BBB [2]. However, a VIP–TFRmAb conjugate results in a 65% increase in hemispheric brain blood flow in the conscious rat following intravenous administration of low doses, 10–20 μg/kg, of VIP [63]. Brain-derived neurotrophic factor (BDNF) is neuroprotective in cerebral ischemia following direct intra-cerebral injection of the neurotrophin in the ischemic region of brain. Intravenous BDNF is not neuroprotective in cerebral ischemia because (i) BDNF does not cross the BBB, and (ii) the BBB is intact in the initial hours after cerebral ischemia when neuroprotection is still possible [64–66]. The intravenous administration of a BDNF–TFRmAb conjugate results in complete neuroprotection of hippocampus CA1 neurons in transient forebrain ischemia [64], and a 65–70% reduction in stroke volume in permanent or reversible middle cerebral artery occlusion [65,66] (Table 1). The reduction in stroke volume

TABLE 1

Therapeutic effects in brain following intravenous administration of peptides, recombinant proteins or non-viral gene medicines attached to molecular Trojan horses

Peptide	Gene therapy	Species	Pharmacological effect	Reference
VIP	–	Rat	Increase in cerebral blood flow	[63]
BDNF	–	Rat	Complete neuroprotection of hippocampal CA1 neurons in transient forebrain ischemia	[64]
BDNF	–	Rat	65–70% Reduction in stroke volume in permanent or reversible middle cerebral artery occlusion	[65,66]
FGF-2	–	Rat	80% Reduction in stroke volume in permanent or reversible middle cerebral artery occlusion	[68]
$A\beta^{1-40}$	–	Mouse	Imaging brain amyloid *in vivo* with peptide radiopharmaceutical	[69]
EGF	–	Rat	Early detection of brain cancer *in vivo* with peptide radiopharmaceutical	[70]
PNA	–	Mouse, rat	Imaging gene expression *in vivo* with antisense radiopharmaceutical	[71,72]
–	EGFR antisense	Mouse	100% Increase in survival time in intra-cranial human brain cancer	[73]
–	RNAi	Mouse	90% Increase in survival time in intra-cranial human brain cancer	[74]
–	TH	Rat	Complete normalization of strialtal enzyme activity in experimental Parkinsons disease	[75]

Abbreviations: VIP, vasoactive intestinal peptide; BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; EGFR, EGF receptor; PNA, peptide nucleic acid; RNAi, RNA interference; TH, tyrosine hydroxylase.

following intravenous administration of the BDNF–molecular Trojan horse conjugate is associated with an improvement in neuro-behavior in animals with experimental stroke [67]. Fibroblast growth factor (FGF)2 is a potent neuroprotective agent when injected directly into ischemic brain. However, in the absence of BBB disruption, intravenous FGF2 is not neuroprotective in cerebral ischemia [68]. The intravenous administration of an FGF2–TFRmAb conjugate results in an 80% reduction in stroke volume in the middle cerebral artery occlusion model [68]. The $A\beta^{1-40}$ peptide is a potential peptide radiopharmaceutical for imaging brain amyloid in AD. Radiolabeled forms of $A\beta^{1-40}$ readily bind to amyloid plaque in autopsy tissue sections of AD brain. However, it is not possible to image brain amyloid plaque following the intravenous administration of an $A\beta^{1-40}$ peptide radiopharmaceutical because this neuropeptide does not cross the BBB [69]. The intravenous administration of an $A\beta^{1-40}$–TFRmAb conjugate in AD transgenic mice enabled detection of brain amyloid by brain imaging [69]. Epidermal growth factor (EGF) is a potential peptide radiopharmaceutical for the early detection of brain cancer because most primary and secondary brain cancers overexpress the EGF receptor (EGFR). However, brain cancer expression of EGFR cannot be detected by an intravenous administration of an EGF peptide radiopharmaceutical because this peptide does not cross the BBB [70]. The intravenous administration of an EGF–TFRmAb conjugate led to the early detection of brain cancer because the reformulated EGF peptide is enabled to cross the BBB [70].

Antisense delivery to brain

Certain antisense radiopharmaceuticals such as PNAs, which hybridize to a specific nucleobase sequence of a target mRNA molecule, could be used as radiopharmaceuticals to image gene expression *in vivo* in the brain, providing the antisense radiopharmaceutical is able to cross the BBB. The intravenous administration of PNA alone does not result in imaging gene expression in brain because the PNA does not cross the BBB [71,72]. However, the intravenous administration of the PNA–TFRmAb conjugate enables *in vivo* imaging of brain gene expression of the caveolin-1α gene in brain cancer [71], or the GFAP gene in brain ischemia [72].

Brain delivery of non-viral plasmid DNA therapeutics

Molecular Trojan horses can deliver non-viral plasmid DNA to brain following intravenous administration with the use of Trojan horse liposomes (THL). The plasmid DNA is encapsulated in a 100 nm pegylated liposome. The tips of 1–2% of the polyethylene glycol (PEG) strands are conjugated with a BBB molecular Trojan horse such as the TFRmAb or the HIRmAb. A 90–100% increase in survival time was observed in mice with intracranial human brain cancer following the weekly intravenous administration of a plasmid DNA encoding either a 700 nucleotide antisense RNA against the EGFR [73] or a short hairpin RNA against the human EGFR [74]. Experimental PD is associated with a >90% decrease in striatal tyrosine hydroxylase (TH) enzyme activity [75]. There is a complete normalization of striatal TH enzyme activity following intravenous administration of a TH encoding plasmid DNA that is encapsulated in THLs and delivered across the BBB and across the neuronal cell membrane with a TFRmAb molecular Trojan horse [75].

Genetic engineering of molecular Trojan horses for BBB delivery in humans

The most potent BBB molecular Trojan horse known to date is the murine HIRmAb [2]. This mAb cannot be administered to humans owing to immune reactions to a mouse protein. Therefore, a murine HIRmAb has been genetically engineered to form either a chimeric HIRmAb [76] or a humanized HIRmAb [77], and both proteins cross the primate BBB *in vivo* at rates comparable to the original murine HIRmAb. The availability of the genetically engineered molecular Trojan horses will enable the future development of large-molecule neurotherapeutics for human brain disorders.

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