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Combinatorial Approaches for the Identification of Brain Drug Delivery Targets

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Abstract

The blood-brain barrier (BBB) represents a large obstacle for the treatment of central nervous system diseases. Targeting endogenous nutrient transporters that transcytose the BBB is one promising approach to selectively and noninvasively deliver a drug payload to the brain. The main limitations of the currently employed transcytosing receptors are their ubiquitous expression in the peripheral vasculature and the inherent low levels of transcytosis mediated by such systems. In this review, approaches designed to increase the repertoire of transcytosing receptors which can be targeted for the purpose of drug delivery are discussed. In particular, combinatorial protein libraries can be screened on BBB cells *in vitro* or *in vivo* to isolate targeting peptides or antibodies that can trigger transcytosis. Once these targeting reagents are discovered, the cognate BBB transcytosis system can be identified using techniques such as expression cloning or immunoprecipitation coupled with mass spectrometry. Continued technological advances in BBB genomics and proteomics, membrane protein manipulation, and *in vitro* BBB technology promise to further advance the capability to identify and optimize peptides and antibodies capable of mediating drug transport across the BBB.

Introduction

Treatment of central nervous system (CNS) disease faces many hurdles. While the drug discovery process itself can prove challenging, a significant bottleneck in CNS therapeutic development has been the restricted CNS delivery of such drugs [1]. The BBB is necessary for the proper function of the CNS; but, the BBB also prevents many small molecule drugs and the large majority of large molecule therapeutics like protein and gene medicines from entering the brain [2]. The BBB comprises specialized endothelial cells that form the vasculature in the brain. These endothelial cells are characterized by tight junctions [3], efflux transport systems, low levels of pinocytotic uptake and a lack of fenestrae resulting in a barrier that is resistant to drug transfer from blood into brain [4].

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Non-Invasive Drug Delivery Via Endogenous Transport Systems

Because the BBB physically separates the CNS from the bloodstream, it is equipped with specialized transport mechanisms that allow the brain to acquire necessary nutrients from the blood. If utilized correctly, these transport mechanisms represent potential delivery portals to the brain. There are three main classes of trans-BBB transport mechanisms: carrier-mediated, absorptive-mediated, and receptor-mediated transport. Carrier-mediated transporters tend to be size and shape selective and hence, limited to drugs that are essentially nutrient mimics, and are not of great utility for large molecule therapeutics [5]. Absorptive-mediated transcytosis is capable of carrying larger payloads into the brain, but as this mechanism is characterized by charged interactions at the BBB, it is intrinsically a nonspecific approach, although it has been studied and used to promote brain penetration [6]. Receptor mediated transcytosis (RMT) pathways, depicted in Figure 1, represent promising targets for drug delivery because payloads ranging from small molecules, to fusion proteins, to liposomes can be ferried across the brain endothelial cells via a specific interaction between a ligand and its RMT receptor [1, 7]. Previous reviews have extensively covered the benefits and drawbacks for the aforementioned delivery strategies [1, 5, 8, 9]. In this review, we will instead focus on the leveraging of high throughput technologies for identification of targeting reagents and their cognate RMT systems, along with the various parameters that direct these screens.

Transferrin Receptor as a Case Study

The transferrin receptor (TfR) is the most well-studied RMT system that has also been co-opted as a brain drug transporter. As such, a discussion of the various brain drug delivery studies involving the TfR helps to illustrate the key parameters to take into consideration when weighing the drug delivery candidacy of RMT systems. Transferrin (Tf), the natural ligand for TfR, is a protein that binds iron. Upon binding the TfR, the iron-bound transferrin can be ferried into the brain as well as other organs by a transcytosis mechanism whereby endocytosed and subsequently transcytosed vesicles are transported across the endothelium leading to iron release into the brain (Figure 1). The capability for such transcytosing vesicles to accommodate cargo up to 100 nm in size [10] make RMT systems particularly suited to the transport of large payloads such as proteins, DNA or nanoparticles. The TfR has been used to deliver payloads to the brain using two distinct targeting ligands, Tf or an antibody that targets the TfR. Conjugation of drug payloads directly to a Tf molecule has been used to successfully deliver drug payloads to the brain [11, 12] however, the TfR is largely saturated with endogenous transferrin under physiological conditions, so the payload-conjugated Tf would have to compete with the endogenous Tf pool, necessitating high concentrations for effective delivery. These factors could raise concerns about side effects because of both the high required dose and inhibition of iron transport by endogenous Tf. One recent study isolated a peptide that can mimic iron and cause Tf to adopt the iron bound conformation after which, Tf can mediate payload transport into the brain [13]. However this approach is still subject to the difficulties discussed above, except that instead of competing with Tf for binding to TfR, the delivery vector would instead be competing with iron for binding to Tf.

Because of the aforementioned competing ligand issues, surrogate ligands in the form of antibodies that target the TfR at a site distal to the Tf binding site have been broadly researched [14–16]. One example is the OX26 monoclonal antibody that recognizes the rat TfR. This molecule has been used successfully to target pharmacologically relevant amounts of drug to the rat brain [14, 17, 18]. However, only 0.44 % ID/g (percent injected dose per gram of brain) of OX26 can actually be detected in the brain [5]. This is partly because the TfR is ubiquitously expressed leading to antibody uptake into peripheral organs. Given the suboptimal uptake, observed pharmacological effects in brain will therefore depend on the potency of the conjugated drug. In addition, the OX26 antibody was not developed specifically for drug delivery and hence its intrinsic binding properties may not be perfectly matched to those required of an efficient antibody delivery vehicle. For instance, Yu et al. recently reported that a TfR targeting antibody's ability to penetrate the mouse brain could be increased by a reduction in affinity for TfR [19]. The idea was that if the offrate of the antibody was too low (i.e. it had a high binding affinity), it would not release into the brain in appreciable amounts (Figure 1, step 5). The subsequent reduction in affinity of the antibody resulted in a ~0.6 %ID/g for the reduced affinity antibody compared to the 0.22 %ID/g of the high affinity antibody at a 24 hour time point [19]. However, to achieve the benefits of lowered affinity, dosing had to be increased substantially because TfR binding (Figure 1, step 1) is also decreased by the lowered antibody-TfR affinity.

This brief review of the TfR RMT system allows for a few key observations regarding the suitability of RMT systems as drug delivery vectors. First, vesicle size is compatible with the transport of larger payloads. Second, the interaction between receptor and ligand can be designed so as not to interfere or compete with the natural ligand. Next, in the case of antibody targeting reagents, the interaction with the RMT receptor can be fine-tuned using antibody engineering techniques to provide improved uptake. Finally, it would be ideal for an RMT system to allow for brain-specific delivery. However, the RMT systems currently targeted, including the transferrin receptor, insulin receptor, and low density lipoprotein receptor-related proteins, among others, are widely expressed in other organs [5, 20], which results in lowered %ID/g and nonspecific uptake throughout the body.

Motivating the Discovery of Novel Transport Targets

The ubiquitous nature of current brain RMT targets has motivated many academic and industrial laboratories to search for alternative RMT systems. An ideal RMT system suitable for use as a brain therapeutic transporter would have three main characteristics. The first would be selective expression at the BBB with zero or comparatively low expression in the peripheral vasculature. The second characteristic would be high RMT abundance at the BBB helping lead to an efficient flux through the endothelial cells. The final characteristic that incorporates aspects of the first two, is that the RMT system would result in efficient payload transport (%ID/g) across the BBB. The deficiencies of the currently known RMT systems compared with such an ideal target has led to the search for novel RMT systems. Moreover, there is evidence that novel RMT systems may exist at the BBB since its unique barrier phenotype likely mandates that it has an enhanced profile of nutrient transporters and RMT systems. Indeed, several studies have suggested that there are unknown receptors (or not known to be expressed at the BBB) waiting to be discovered. In early BBB genomic

studies, suppression subtractive hybridization (SSH) was used to show that many of the differentially expressed cDNA transcripts at the BBB compared to liver and kidney tissue, function in transport or were of unknown function [21]. In a later study performed by Enerson and Drewes [22], a serial analysis of gene expression (SAGE) approach indicated that many brain microvessel transcripts were enriched compared with total brain and only 31% were genes of known function [22]. These findings combined with recent data from Daneman et al. [23] which showed a large number of transporters upregulated at the BBB in a genome-wide comparison of endothelial cells of mouse brain, liver and lung, indicate there is a high likelihood for the existence of other BBB RMT systems that have not yet been exploited for drug delivery.

The search for these RMT systems has been under way for well over a decade and this review seeks to cover the combinatorial methodologies and results in the context of key parameters governing RMT fitness as a drug delivery target. Initially, the discussion will focus on combinatorial methods for lead targeting molecule discovery (e.g. peptides, antibodies, and viruses), and the important technical aspects involved, such as display scaffold and screening platform. The second portion of the review will focus on the difficult task of identifying the BBB RMT system recognized by the targeting molecules.

Combinatorial Screening Methodology

Finding and identifying unknown BBB RMT systems can be thought of as the proverbial search for a needle in a haystack, only in reality it is more like finding two matching needles in two separate haystacks. The two needles in this case would be the RMT system and its cognate targeting ligand, while the two haystacks would be the complex protein composite expressed in the plasma membranes of BBB endothelial cells and a library of cognate targeting ligands, respectively. The combinatorial approach for novel transporter discovery involves screening the library of targeting ligands against BBB endothelial cells, the basics of which are depicted in Figure 2. A library of targeting ligands is generally a peptide or antibody library, comprised of on the order of 10^7 - 10^{10} members. Inherent to the library is a “display” platform, such as phage or yeast, which directs peptide or antibody biosynthesis and display on individual phage or yeast particles. Importantly, each phage or yeast particle displays a single peptide whose genetic material is harbored by the same particle, creating the critical phenotype to genotype linkage that allows separation, amplification, and sequence identification of desired clones. Then, the BBB endothelial cell “substrate” containing the desired spectrum of RMT systems must be chosen. This endothelial substrate is presented to the library of targeting ligands, and the library mixture is allowed to bind/internalize/transcytose, depending on the screening format. This is followed by a series of wash steps to remove non-specific targeting agents from the library pool. The members of the library that are still associated with the BBB cells are subsequently recovered and amplified. This entire process represents one round of screening. Through multiple repetitions of the screening process, the library will become enriched for peptides or antibodies that interact as desired with the BBB endothelial cells. After completing the screen, the phenotype of the recovered clones is individually validated. While this process pulls one needle out of the haystack, isolating the targeting ligands from a highly diverse library, the actual identity of the other needle, the RMT system, needs to be determined

using a different toolkit. In fact, antigen identification is oftentimes the most difficult component of combinatorial screens performed on cell-based substrates. Below, we will discuss the various forms of BBB-targeted combinatorial screens that have been described in the literature, and follow that with methods that can be used for antigen identification. First, however, we provide a brief discussion of the technologies involved to inform the BBB applications.

Antibody-based Libraries

There are a number of different protein scaffolds that can provide the binding motifs necessary for a BBB targeting reagent. Restricting to those previously used in BBB studies, the protein scaffolds can be broadly grouped as antibody-based, peptide-based and virus-based. Antibody-based libraries are usually created by amplification of the variable regions of immunoglobulin genes isolated from animal or human sources although synthetic diversity can also be introduced, as reviewed by Bradbury et al. [24]. Many different assembled antibody constructs have been employed ranging from single variable domain molecules to full IgG antibodies (Figure 3). Most libraries employ at least the antibody variable heavy chain (VH) and antibody variable light chain (VL) connected with a linker to form a single-chain antibody (scFv). Another format called a single domain antibody (sdAb), is constructed from camelid mRNA and contain only VH genes [25, 26]. ScFv and sdAb libraries can be generated from immunized hosts or from their nonimmune repertoire. Nonimmune libraries tend to be used when the antigen is unknown, as in most brain targeting screens. Immunized libraries are used mainly if one desires an antibody bias towards a specific, known antigen or a collection of antigens. Importantly, immunized libraries tend to result in antibodies with higher affinities to the antigen due to the *in vivo* maturation that occurs prior to repertoire isolation [27], while nonimmune libraries tend to yield lower affinity lead molecules, an important consideration when considering the impact of antibody properties on the efficiency of RMT-based delivery.

Peptide-based Libraries

There are also many forms of designed, binding peptide libraries that typically vary in peptide length and conformation. For example, they can be presented as linear peptides from 7 to 15 amino acids long [28, 29], or presented as conformationally constrained peptides through cysteine cyclization [30]. Peptide libraries are especially useful because of the ease of production and their correspondingly high diversity. In general, higher and more tunable binding affinities can be achieved using antibodies compared with peptides, but peptides are often used in a multivalent context for delivery applications (e.g. decorating nanoparticles or liposomes) to combat the affinity ceiling [31]. Virus-based libraries are conceptually similar to peptide-based libraries and will be discussed further below.

Library Display Platforms

In the search for novel peptides and antibodies that target brain RMT systems, a number of display platforms have been employed (Figure 3). Thus far, the most widely used platform is phage display, but yeast display, and more recently adeno-associated virus (AAV) have also been used to identify brain targeting molecules. The choice of display platform is of great

importance because they each have their associated advantages and disadvantages for BBB-based screens.

Phage Display

To date, phage display has been the predominant display platform used for combinatorial screens involving brain endothelial cells. Filamentous bacteriophage are small “toothpick” like viruses that infect F pilus positive *E. Coli*, and some other types of bacteria [32]. The ability to take recovered phages having the desired phenotype and infect and amplify in *E. Coli* contributes to the simplicity of most phage-based protocols. The most common bacteriophage used are filamentous phages, either M13, fd, or f1 [24], although other bacteriophages such as T7, or λ phage have been used successfully as well [33]. Peptides or antibodies are displayed as fusions to phage coat proteins, particularly the pIII protein that is present at one end of the phage particle depicted in Figure 3b. For a more in depth review on the methods and coat protein fusions used in displaying foreign peptides on filamentous phage see Tohidkia et al. [34] or Rakonjac et al [31]. While using wild type phage in conjunction with a phagemid encoding the pIII fusion results in predominantly 0-1 copies of fusion protein displayed on the phage particle, modification of the phage genome itself can lead to multivalent display of 3-5 copies [31, 35]. There are two main characteristics that make phage display a particularly useful display platform for BBB-based screens. The phage themselves are small (930 nanometers in length and 6.5 nanometers in diameter [32]) which makes the phage platform amenable to internalization or endocytosis screening and *in vivo* screening [36]. Multivalent display in the phage format can promote more efficient receptor clustering and endocytosis [35], and is therefore most useful for RMT screens, however the monovalent format can be useful for recovering targeting molecules with a higher affinity. Moreover, the fact that phage can be internalized after binding its putative RMT target, gives the advantage of being able to screen directly for an internalizing or transcytosing phenotype, as opposed to screening for binding only. In addition, *in vivo* screening can allow access directly to the expressed protein composite in blood vessels. The *in vivo* phage screening method was initially developed in 1996 by Renata Pasqualini and Erkki Ruoslahti in a landmark study where they injected mice with various phage displayed peptide libraries and discovered three peptide motifs that localized to the brain and two motifs that targeted the kidney [30]. Phage display tends to suffer from non-specific binding of phage to the target cells, although this can oftentimes be overcome by limiting the number of screening rounds and by oversampling of recovered clones. In addition, because it employs rudimentary bacterial protein folding mechanisms, phage display has been shown to have a more limited functional diversity in antibody libraries compared with yeast display [37].

Yeast Display

As a eukaryotic alternative to phage display, and in part to help overcome the protein folding handicap of bacteria and phage display, a robust display platform that relies on the display of peptide libraries on the surface of yeast was developed [38]. Antibody fragments are fused to the Aga2p mating protein to allow surface display of on the order of 30,000-100,000 copies per cell [38], a cartoon of which is shown in Figure 3. Importantly, it has been shown that utilization of the eukaryotic folding machinery can lead to higher fitness libraries, thereby compensating for the smaller library size achievable with the yeast display platform [37].

Important to BBB cell surface-based screens, the highly multivalent antibody display increases the likelihood of finding lead molecules because even lower affinity antibodies can be recovered as a result of avidity effects [39]. Most yeast strains used for display are also flocculin deficient, which leads to a low level of background binding when screened on mammalian cells [39]. As another advantage, the use of yeast display facilitates the rapid downstream evaluation of antibody-antigen properties including quantitative affinity determination, antibody specificity, and antigen cloning [40, 41]. The primary drawback of the yeast display system for BBB-based screens is that the size of yeast (5 micron in diameter), prevents them from being used directly for internalization or *in vivo* screens. Instead, binding screens are performed with secondary evaluation of recovered antibodies for endocytosis or transcytosis capacity [40].

Adeno-Associated Virus

While AAV has long been researched as a tool for gene delivery, it recently has been exploited in BBB-based screens [42]. AAV is a small, ~25 nm, diameter member of the parvovirus family that is replication deficient, leading to its popularity in gene therapy applications [43]. Although AAV is not a display platform in the classical sense, like phage or yeast display, a number of recent studies have used combinatorial techniques to modify the surface of AAV particles (i.e. the displayed proteins on the viral coat, Figure 3) leading to the adoption of altered tropisms, the ability to preferentially transduce certain cell types [44, 45]. There are two established means for achieving altered tropism. One is called serotyping, and the other is engineering of virus coat peptides. Each of the nine known AAV serotypes appears to have different cell tropisms. The AAV9 serotype has been particularly interesting to the brain drug delivery community because it shows evidence of brain penetration in young or developing brains [46]. In other studies, the viral coat proteins were engineered by shuffling in an attempt to find AAV variants with preferential brain tropisms [47]. One significant limitation is the difficulty in achieving the fine specificity of AAV targeting. While tropisms can and have been modified, it is very hard to limit viral transduction to only one targeted cell type, although some progress has been made [48, 49].

Screening for Brain Targeting Agents

After combining the desired protein scaffold and display platform to meet BBB screening goals, the next component is to select an appropriate brain endothelial screening substrate. These substrates can come from the list of BBB cell lines, primary BBB cultures, isolated plasma membrane preparations, or the *in vivo* vasculature. Below we organize the combinatorial screens that have been used to search for BBB targeting and transporting molecules in the context of these various screening substrates. These studies have also been cataloged in Table 1.

In Vitro Screens

The first approaches used to identify putative BBB targeting antibodies and peptides employed various *in vitro* BBB models to screen for peptides that bind, endocytose or transcytose the chosen model. After the *in vitro* screening steps, it is important to perform *in vivo* characterization and antigen identification to help confirm *in vivo* relevancy.

As an example of a BBB cell line-based screen, yeast displayed antibodies were used to generally profile the surface of BBB endothelial cells, and this initial binding screen was followed by a second step that explored the endocytosis potential of the selected antibodies [40]. A nonimmune library of $\sim 1 \times 10^9$ human scFv in the yeast display format [50] was panned against the RBE4 cell line of immortalized rat brain endothelial cells for 5 rounds. The screen resulted in 34 unique RBE4-binding antibody clones that, based on CDR homology, could be clustered into 16 binding classes. Four of the 16 classes of antibodies displayed the capability to both bind and internalize into RBE4 cells, and one particularly promising antibody, scFvA, was shown to bind the *in vivo* BBB by tissue section and freshly isolated capillary immunohistochemistry [40] (Table 1). Studies to determine the scFvA transcytosis potential and target RMT system are underway. A second related example of using yeast to discover BBB relevant antibodies employed the same yeast displayed scFv library to screen against plasma membrane-biotinylated RBE4 cell lysates [51]. Eleven unique antibodies, in a non-exhaustive sampling, exhibited binding to the biotinylated lysate and the membranes of intact RBE4 cells (Table 1). Although validation of the identified antibodies remains to be performed, the utility of this approach is that it can likely sample a larger repertoire of the plasma membrane than panning approaches [51]. Both of the aforementioned studies employed an immortalized brain endothelial cell line, and given that there are many shortcomings of BBB cell lines such as low junctional integrity and attenuated transporter expression [52], many relevant RMT systems may be missed in such screens. Moreover, the cells originate from rat and thus as an alternative, it may also be possible to use an immortalized human BBB cell line such as the recently created hCMEC/d3 line [53] to perform similar screens and identify antibodies having greater likelihood to bind human target proteins. Finally, although a secondary screen of the antibodies can be used to determine brain specificity and internalization potential, it is not intrinsic to the screening process.

These factors were taken into consideration in the next example where primary brain endothelial cells along with subtractive phage panning were employed in an effort to increase *in vivo* relevance and BBB specificity, respectively. As mentioned earlier, one of the ideal requirements for a brain-penetrating antibody is brain specificity. One way to select for brain-specific targeting antibodies is by incorporation of a library pre-subtraction step. In this way, a large naive library (5×10^8 members) of camelid single domain antibodies (sdAbs) displayed on fd phage in a multivalent format were screened against a primary human endothelial cell model for binding and internalizing phage clones [26]. However, prior to screening on the primary human endothelial cells, the sdAb library was first pre-subtracted by incubating on human lung endothelial cells to remove clones that bind common endothelial antigens. This helps to facilitate recovery of clones that have specificity for brain endothelial cells. Two full-length sdAb phage clones were isolated at the end of the panning designated FC44 and FC5. Subsequently, solubly expressed sdAbs were shown to interact with the BBB of mice *in vivo*. These BBB targeting clones also showed significantly less binding to the lungs *in vivo* than control molecules, indicating some benefit to the pre-subtraction step built into the screening procedure. Since its discovery, FC5 has been used fairly extensively to deliver a variety of imaging and therapeutic cargoes to the brain

including the imaging contrast agent cy5.5 and the MRI contrast agent Gadolinium-DPTA as well as the therapeutic doxorubicin [26].

While the *in vitro*-based screening approaches are simpler than the *in vivo* panning approaches discussed below, putative unknown RMT systems might be missed. As alluded to earlier, it has been shown that primary BBB cells and immortalized BBB cell lines have significantly different gene and protein expression profiles than those found *in vivo*, with a number of BBB-selective genes being downregulated [54, 55]. These findings imply that unknown brain specific RMT systems also have a high likelihood of being significantly downregulated or absent *in vitro*.

In Vivo Screens

In order to gain access to the receptors potentially missed by *in vitro* approaches, several screens have recently been performed directly *in vivo*. These screens have most often been performed by intravenous injection, but *in situ* brain perfusion of the combinatorial libraries has been used as well. *In vivo* screens tend to be more technically difficult because of the complexity associated with a living animal, the extra steps necessary to isolate the clones of interest from the brain during screening rounds, and the relatively small population of selectable cells.

A pair of *in vivo* screening examples employed phage displayed peptide libraries to screen for BBB homing peptides in mouse following intravenous injection [56, 57]. There exist at least three major challenges when performing *in vivo* screens with phage-based libraries. One is the loss of rare, potentially promising clones present in the initial library to organs other than the brain. The second is that there tends to be a high level of nonspecific phage remaining in the brain at the time of brain harvesting and clone recovery, masking desired clones. The third, which is related to the first, is the rapid loss of library constituents to the liver and spleen as 90% of injected phage can accumulate in the liver and spleen within the first minute after injection [58]. These two studies sought to partially address the second problem by using an experimentally optimized blood circulation time that maximized the brain to blood ratio of phage, increasing the likelihood of identifying brain targeting phage [56]. Interestingly, this time point was found to be at 24 hrs when a large component of the initial library was likely already cleared by liver and spleen, and hence, most other *in vivo* binding screens have been performed on the 3-15 minute time scale [28, 58, 59]. The first study, used the Ph.D. linear 12mer phage display peptide library, and after four rounds of *in vivo* screening, a 250-fold increase in the amount of phage recovered from the brain was observed. Twenty peptides were sequenced and one peptide, designated pep TGN, was found to make up 60% of the pool. Marginal TGN-displaying phage binding was observed in cryopreserved brain sections after intravenous injection. The peptide was then used to decorate the surface of PEG-PLGA nanoparticles to characterize its ability to deliver a dye-based payload to the brain. The pep TGN decorated nanoparticles were shown to internalize into the bEnd.3 mouse endothelioma cell line, as well as localize to mouse brain when injected *in vivo*, although no attempt was made to examine whether or not the particles actually crossed the BBB or remained sequestered bound to, or internalized in, BBB endothelial cells. TGN-modified nanoparticles also showed uptake in the liver and spleen,

which could have been a result of nonspecific uptake via the reticuloendothelial system. The second similar study instead utilized the constrained peptide library, Ph.D-C7C, which is a disulfide-bonded cyclic heptamer library [57]. As in its sister study, four rounds of *in vivo* screening were performed, but at the end of the fourth round, the phage titer had only increased by approximately 13-fold. Twelve clones were sequenced at the end of round four, with one sequence designated PepC7 being predominant. Very infrequent brain binding of the PepC7 displaying phage was observed by immunohistochemistry after intravenous injection, and when the peptide was conjugated to Cy5.5 for *in vivo* imaging it had some brain selectivity although not as strong as the aforementioned TGN peptide. While it is unclear whether the peptides in either of these two studies actually enter the brain parenchyma, it appears that they are at least binding the brain vasculature, and they warrant more characterization in terms of brain penetration and targeted BBB antigen identification. Even if these peptides ultimately are shown not to enter the brain parenchyma, it is conceivable that they could be used in other applications, such as decorating AAV particles for gene delivery as was done in Chen et al. [28] and discussed in the disease state section below.

As mentioned briefly above, library mining directly *in vivo* suffers from the rapid scavenging of phage virions from the bloodstream by organs other than the target organ [58]. While phage disappearance from the bloodstream has traditionally been attributed to organ scavenging, the innate immune system may also play a significant role through specific inactivation of peptide displaying phage [60]. Regardless of the mechanism, this potential for loss poses a difficulty when one considers that there may be only 1-10 copies of a desired phage clone injected into an animal. In this situation, a putative binding or transporting phage clone with a low representation in the original library could be completely removed from the blood stream before it ever gets to the brain, thereby effectively reducing the diversity of the library. One approach to circumvent this problem was employed by Van Rooy et al. [29] where a 15 amino acid peptide phage display library was administered to the mouse by *in situ* brain perfusion as opposed to intravenous injection. In this particular form of *in situ* brain perfusion, the phage display library is introduced into the left heart ventricle where it then travels through the aorta and ultimately to the brain. Usually the descending aorta is clamped as well to force the phage solution into the brain and prevent it from contacting peripheral organs, somewhat isolating the brain vasculature [29]. After perfusion of the library, the brain was recovered and the phage amplified for a total of three rounds of screening. Individual clones were further analyzed and two named GYR and GLA preferentially bound the hCMEC/D3 human brain endothelial cell line compared with human umbilical vein endothelial cells. Finally, the two phage clones were individually perfused and were recovered from brain at a 5-6 fold higher level than the negative control phage. In a later study the same researchers attempted assess the usefulness of these peptides for drug delivery by conjugation to nanoparticles [61]. They found that producing the peptides synthetically resulted in loss of the hCMEC/D3 cell binding phenotype. However the binding phenotype was restored when the peptide was produced as a fusion to the phage pIII protein, and then incorporated into the nanoparticles. This illustrates the importance of the display platform for the induction of peptide conformation or binding properties. Further

binding and transport validation of GYR and GLA is necessary to determine their usefulness for brain drug delivery applications.

Importantly, when using an *in situ* perfusion screening method, the phage only have a short time (~5 minutes depending on perfusion speed and volume of the library) to contact and bind their antigen, which could bias the screen for the highest affinity binders or the highest abundance RMT targets. Secondly, this timeframe is too short to have substantial phage internalization at the BBB endothelium, meaning that it is generally not possible to screen for brain penetrating peptides per se, only for those that bind. However, since binding is a prerequisite for internalization and transcytosis, such screens do not preclude the isolation of brain penetrating peptides. While intravenous and *in situ* methods are the two routes of administration primarily used to screen libraries *in vivo*, at least two alternative routes have been explored. Wan et al. isolated peptides from a phage displayed library that penetrated the brain via the olfactory pathway using intranasal administration, although brain penetration appeared somewhat limited [62] (Table 1). Also, in a interesting study performed by Gonzalez et al. in 2011 [63], phage display was utilized to isolate three clones from a cyclized heptamer library that internalize into the choroid plexus (CP) epithelia of rodents (Table 1). They showed strong evidence for the binding and internalization of these clones both *in vitro* and *in vivo* by intracerebroventricular (i.c.v.) injection. While an invasive injection is required, the general screening approach could in principle also result in identification of peptides that target the CP epithelia via the bloodstream [63].

Disease State Brain Endothelium Screens

Mining for RMT targets present at the normal BBB is important as many of these targets are also likely present at some level under disease conditions. However, in diseased brain, the BBB protein expression profile can differ from that found in a healthy brain [64]. Chen et al. sought to take advantage of this differential expression profile to isolate peptides that specifically target the BBB of animals having lysosomal storage disease [28]. The researchers screened an intravenously administered phage display heptapeptide library *in vivo* on healthy mice, as well as two types of lysosomal storage disease model mice: mucopolysaccharidosis type VII (MPS VII) and late infantile neuronal ceroid lipofuscinosis (LINCL). They found that different peptide motifs appeared in the final rounds of screening for each model, and these separate motifs showed specificity for the model they were selected on. After screening, the peptides were inserted into an AAV capsid protein (Figure 3) to retarget an AAV that harbored genes encoding key enzymes that are lacking in the lysosomal storage disorders. The redirected AAV vectors transduced brain endothelial cells, and directed expression of the encoded enzymes partially mitigating disease symptoms. These data suggested that transduction of endothelial cells can lead to secretion of pharmacologically relevant amounts of therapeutic enzyme into the brain parenchyma. This is particularly interesting because it shows that in some cases the BBB does not have to be traversed in order for relevant brain disease therapy, but that the brain vasculature itself can be used as therapeutic reservoir.

The seizure compromised blood-brain barrier has also been subjected to a combinatorial targeting screen. In a study by Gray et al. [47], the capsid-encoding DNA from AAV

serotypes 1-5, 8 and 9 was shuffled to create a combinatorial AAV library having engineered capsid proteins. The resulting library was intravenously injected into seizing rats, and the AAV DNA was recovered by PCR from the seizure compromised portions of the rat brain. Two of the four isolated AAV clones, designated 32 and 83, exhibited significant transduction of neurons at locales of seizure-compromised BBB while showing negligible transduction elsewhere. The recovered engineered capsid of these clones was a mixture of AAV1, 8, and 9. These AAV particles had the added and unintended bonus of gaining a level of specificity to the brain. Compared to parent serotype controls, these two clones did not significantly transduce the liver, and exhibited decreased transduction in most other organs.

While the two diseased brain screens are illustrative of the general approaches, there have been two additional screens designed to identify molecules that target diseased state brain endothelium. One such study was performed by Hong et al. in 2008 [65], in which a cyclic peptide that homes to ischemic stroke-compromised brain tissue was isolated from a T7 phage displayed library. Another study performed by Roodnik et al. in 2010 [59] was particularly interesting in that unlike the other studies listed thus far, it employed an immunized sdAb library rather than a nonimmune antibody pool. An sdAb library recognizing glioma tissue was screened *in vivo* after intravenous injection to identify an sdAb that preferentially binds brain tumor vasculature over normal brain vasculature. As an aside, it is interesting to note that almost all reported *in vivo* screens using phage display for both BBB and other applications have employed peptide-based display libraries. While antibodies may be preferable for certain delivery applications, we are aware of several attempts to screen nonimmune antibody phage display libraries *in vivo* with limited success. Thus, the study by Roodnik et al. might suggest that an immunized library is key to successful *in vivo* antibody screens.

Fusing Combinatorial Screens with Rational Design

In some cases, rational design can be used to help direct combinatorial screens. An elegant example of this approach was a study performed by Demeule et al. in 2008 [66]. In this study, a ligand-based motif that targets low density lipoprotein receptor-related protein (LRP) was used as a lead peptide. A small library of 96 variants was generated from the lead peptide that was then screened using two tiers of discrimination. The first was the measurement of peptide transcytosis through a transwell model of the BBB in which primary bovine brain endothelial cells were co-cultured with rat glial cells. Peptides were placed in the apical chamber, and allowed to transcytose. Those peptides that had the highest percentage transcytosis were then validated with an *in situ* brain perfusion experiment to test the capability of the peptides to interact with the mouse BBB, leading to the identification of Angiopep-1. Since its discovery, Angiopep-2, a single amino acid variant of Angiopep-1, has been developed and used to target various payloads to the rodent brain *in vivo* including dendrimers [67], liposomes [68] and carbon nanotubes [69]. Moreover, Angiopep-2 has recently been used conjugated to paclitaxel for clinical treatment of advanced solid tumors, and several patients had shrinkage of brain metastases [70].

Identification of Targeted Antigens

As a consequence of the nature of the combinatorial screens described thus far, a targeting peptide or antibody is discovered without prior knowledge of the actual identity of the targeted BBB RMT system (Figure 1). Thus, the next important step is to identify the RMT system before employing the targeting peptides or antibodies for brain-targeted drug delivery. While the peptides, antibodies, and viruses resulting from screens often generate the most excitement, a perusal of the literature makes it clear that most of the targeted BBB proteins remain unidentified. Target identification is a laborious process, and several classical methods as well as newly developed methods have been employed with the hope of identifying targeted BBB receptors. One of the overarching challenges is that the BBB targets are often membrane proteins having low abundance. Moreover, given their membrane spanning domains, the target proteins suffer from insolubility and may be post-translationally modified. These factors add up to proteins that are not well-matched to a standard protein identification procedure such as separation and mass spectrometric peptide sequencing. Below, we detail several possible approaches that have either been used for BBB membrane protein studies and could be adapted for target identification, or have been used to successfully clone a BBB target resulting from a combinatorial screen.

Immunoprecipitation and Mass Spectrometry

Although BBB RMT targets are membrane proteins, classical immunoprecipitation techniques can be successful in isolating small amounts of protein. Subsequently, tandem mass spectrometry (MS/MS) techniques can be used to sequence the immunoprecipitated membrane protein for *de novo* RMT target identification. There are four major steps to identify an antigen in this way: preparation of the source material containing the candidate RMT receptor, binding of the antibody to its RMT target in the source material, immunoprecipitation of the RMT target from the remainder of the source material, and finally use of MS/MS to sequence the purified RMT target [71]. Since antibody-based immunoprecipitation techniques have been widely practiced for decades, we will focus on recent progress where antibodies directly in the yeast or phage display format have been shown to be compatible with immunoprecipitation and MS/MS techniques.

An RMT protein can be pulled out directly from detergent-solubilized cell lysates using antibodies expressed on the surface of phage or yeast. This approach obviates the need for antibody reformatting, expression, and purification prior to traditional immunoprecipitation. For example, Poul et al [36] identified an scFv (H7) that bound to an internalized tumor antigen by performing an internalization screen using a phage display antibody library. First H7 had to be subcloned, bacterially produced and purified. H7 was then bound to Ni-NTA agarose beads and used to precipitate its antigen from the biotinylated cell lysate. The antigen for H7 was then determined to be the TfR by N-terminal protein sequencing and this identity was confirmed by Western blotting with an anti-TfR antibody. Alternatively, to avoid the laborious antibody production steps, particularly if the cloning of many potential RMT antigens is desired, the antibodies or peptides can be used directly in the phage display format by crosslinking the phage and creating an affinity matrix large enough for centrifugation and antigen recovery [72]. After antigen elution, the antigen can then be

identified by N-terminal sequencing or MS/MS approaches. In a somewhat analogous approach, yeast surface display immunoprecipitation (YDIP), was recently developed by Cho et al. [41] to isolate and characterize membrane protein antigens. YDIP takes advantage of the significant number of peptide copies or antibody copies (30,000-100,000) displayed on the yeast surface to directly immunoprecipitate target antigens from cell lysates. Importantly, clones identified in a combinatorial screen can be used directly in the yeast display format without any modification to isolate, characterize and identify membrane protein targets from brain endothelial cell lysates. As an example, the YDIP technique was used to clone the membrane protein antigen for one of the scFvs isolated in the aforementioned yeast biopanning study discussed in the *In Vitro* Screens section [40]. Yeast displayed scFvJ was used to immunoprecipitate its target antigen from detergent-solubilized lysates of the RBE4 rat brain endothelial cell line. The antigen was separated by SDS-PAGE, digested with trypsin and analyzed by LC-MS/MS to determine that the membrane protein targeted by scFvJ was neural cell adhesion molecule (NCAM). Interestingly, NCAM has been shown to internalize by clathrin-dependent endocytosis mechanisms [73], suggesting the possibility that NCAM could be an RMT-like target, particularly at the tumor BBB [74, 75].

Mechanistic transport analysis can also help elucidate the type of transporter bound by a peptide or antibody identified in a combinatorial BBB screen. For instance, the FC5 sdAb selected by Muruganandam and discussed earlier in the *In Vitro* Screens section has been analyzed quite extensively [26]. FC5 was shown to co-localize with clathrin but not with caveolin-1 by immunocytochemistry and Western blotting, and pharmacologic inhibitors also indicated a clathrin-dependent mechanism of transcytosis [76]. Later, the receptor targeted by FC5 was determined to be a membrane protein known as TMEM-30A, a neuraminidase-sensitive, $\alpha(2,3)$ -sialo-glycoconjugate, expressed on the luminal surface of brain endothelial cells [77].

Expression Cloning

While immunoprecipitation and MS/MS methods are powerful tools to identify targeted RMT receptors, they are not always successful. In particular, problems stemming from RMT protein insolubility or protein abundance continue to limit the effectiveness of these approaches. For these cases, expression cloning can be an option (Figure 4). An antibody identified in a BBB screen can be used directly as the probe to extract the RMT target antigen from a BBB cDNA library expressed in a mammalian host cell. Since the membrane proteins are expressed by a mammalian host using a strong promoter, issues regarding protein solubility and abundance are diminished. As a study relevant to the BBB, a multiplex expression cloning method was developed to identify membrane protein antigens targeted by a BBB membrane protein-specific polyclonal antibody (BMSPA) [78]. A BBB cDNA library was generated from bovine brain microvessels and transfected into HEK293 cells for BBB membrane protein expression (Figure 4, step 1). The HEK293 cells ectopically expressing BBB proteins were then probed with the BMSPA (Figure 4, step 2), and clones expressing a BBB membrane protein recognized by an antibody in the BMSPA polyclonal antibody mixture were isolated by flow cytometry and the harbored cDNA was sequenced (Figure 4, steps 3-7). In this way, 30 BBB membrane proteins targeted by the BMSPA were

identified [79]. The identified proteins display significant diversity in structure, function, and *in vivo* expression level, indicating this approach could be useful for cloning of RMT antigens using antibodies generated from the combinatorial screens.

Although the above expression cloning example employed mammalian cells as the host cells “presenting” the BBB cDNA library, there are other hosts available that can be used in an analogous way. The cDNA library could instead be expressed on the surface of phage [80, 81] or yeast [82, 83] and the antibody of interest used to identify the phage or yeast that harbor the targeted BBB cDNA. Although there are a variety of methods to clone an antigen targeted by an antibody or peptide that is a product of screening, to our knowledge NCAM and TMEM-30A are the only BBB targets resulting from combinatorial screens that have been identified. In addition, target identification is particularly challenging for peptide targeting reagents as they tend to be lower affinity and thus, are less effective when used for immunoprecipitation or expression cloning procedures. In some cases however, a database search of peptides resulting from combinatorial screens may indicate a sequence or structure similarity to a naturally occurring protein. Such similarity may suggest that the peptide acts as a ligand mimic, and if the natural ligand-receptor pairing is known, the target of the peptide can be validated [84].

Conclusions and Future Opportunities

While pioneering work with known RMT-systems has led to adoption of RMT as a potential portal to the brain, the continued development of targets such as the TfR may be limited by receptor ubiquity and the relatively low amounts of payload that cross the BBB. Thus, in the last decade there has been substantial progress made in bringing combinatorial techniques to bear on the BBB problem with the goal of discovering new lead molecules targeting different RMT systems. As described above, screens on various brain endothelial cell platforms including *in vitro* cell culture, endothelial cell lysates, *in vivo* platforms, and even various disease states have shown promise. The problem of facile and rapid antigen identification however, still remains as a major hurdle. Below we close by briefly mentioning a few areas where technical progress in related areas may spur future growth in the application of combinatorial methods for circumventing the BBB.

Exploring “omics” Techniques to Identify Candidate RMT Systems

As described thus far in this review, the combinatorial screen is used to simultaneously identify the targeting antibody or peptide and the cognate transport system. A variety of elegant screening paradigms such as the use of subtraction techniques have been used to help identify targets that may have BBB specificity. Another possibility moving forward is to identify candidate BBB-specific targets by mining genomic and proteomic data sets for those genes and proteins that appear to be substantially differentially expressed at the BBB, or at least expressed in high abundance. Subsequently, the combinatorial screen as depicted in Figure 1 can be performed replacing the cellular substrate with the specific target(s) identified in the omics study. As one relevant example, SSH has been used to compare brain endothelial cells to those of other vascular beds such as heart, liver and kidney [85–87]. Several interesting membrane proteins were identified with unique expression in the brain,

such as BBB specific anion transporter type 1 (BSAT1), which was further cloned by Chu et al. [87] from the rat brain cDNA and classified as an organic anion transporter in rat brain. In a much more comprehensive fashion, many BBB-enriched transcripts compared with lung and liver endothelium were recently identified by gene microarray [23]. Transporters such as *LTIH5*, *APCDD1*, *ABCB1A*, and *SLCO1C1*, had high expression level and selective brain expression compared to liver and lung. From a proteomic standpoint, of the 30 BBB membrane proteins identified in the aforementioned multiplex expression cloning method, five of them were brain selective when compared to the liver, kidney, lung and heart [79]. Proteomic analysis of the BBB using mass spectrometry can also help identify BBB-enriched proteins, including those enriched in disease states. For instance, several membrane proteins have been identified in the diseased brain that have higher expression levels than those in normal brain, such as Igfbp7 and SPARC in glioblastoma multiforme [88] and Aquaporin-4 and Laminin- α 1 in stroke-prone brain [89–91]. Overall, while these examples are unlikely to be RMT systems, they indicate that uniquely expressed proteins at the BBB can be identified using omics technologies. Thus, more information, such as transport capability, transport direction, biological function, and expression level in the brain, should be weighed before moving forward with the development of peptides, antibodies or viruses against BBB targets identified through omics approaches. One approach to compare the abundance of candidate BBB transport systems has been pioneered by the Terasaki group which makes use of a tandem mass spectrometry approach to quantitatively compare ABC transporter and junction protein expression in various species at different ages [92, 93]. Finally, it would be useful to identify RMT systems that have regio-specific expression within the brain microvasculature to better target medicines for disease conditions that are not affecting the full brain. Importantly, gene expression at the normal BBB differs by brain region and by vessel type, indicating the possibility of regio-specific RMT expression. As described by the Pachter group in a series of publications, laser capture microdissection can be combined with various genomic and proteomic methods to examine differential gene expression within the microvasculature of the brain [94].

Advances in *In Vitro* BBB Technology

Recent advances in the area of *in vitro* BBB models could also accelerate the discovery of new RMT systems. As discussed previously, the primary limitations of the screens performed on *in vitro* models is the departure in gene and protein expression profile compared to the *in vivo* situation. Technically speaking, however, screens using *in vitro* systems tend to be easier, they allow for fine-tuning to include aspects such as subtraction and tissue specificity, and they avoid the off-target biological complexities associated with *in vivo* systems. Thus, improvement in the *in vitro* BBB models themselves could result in access to heretofore inaccessible RMT systems. A non-exhaustive sampling of recent advances in *in vitro* BBB technology illustrates a few variables that may have important roles, such as multicellular co-cultures, flow, and BBB model tightness. Since the cellular communication between BBB endothelial cells, astrocytes and pericytes affects the barrier integrity and protein expression at the BBB, Nakagawa et al. [95] compared various multicellular *in vitro* BBB models to determine those models with the most desirable characteristics. They found that a triple co-culture model, employing BBB endothelial cells, astrocytes, and pericytes, best mimics the *in vivo* BBB with highest tight junction protein

expression and barrier function compared with endothelial mono-culture or single cell type co-culture. Also, most BBB models are static models, but it is known that endothelial cells including those of the BBB respond positively to the shear forces of flow. *In vitro* BBB models incorporating pulsatile flow have been developed and the BBB properties move towards the *in vivo* situation [96–98]. However, the comparatively large volumes intrinsic to these models may make screens difficult from a clone recovery standpoint. To this end, Booth and Kim developed a microfluidic model of the BBB which has both a dynamic flow environment and a comparatively thin culture membrane that can be used to assay transport across the BBB [99]. Finally, Lippmann et al. recently reported an *in vitro* BBB model derived from human pluripotent stem cells [100]. This very scaleable human BBB model has robust tightness, a key aspect to a combinatorial transcytosis-based screen. Each of these models may offer the opportunity for additional screens and the discovery of new lead molecules.

Advances in Protein Engineering Against Membrane Protein Targets

An important consideration when evaluating the output of a combinatorial screen is that lead molecules are being generated. They should not be considered as optimized for targeting or transport. Thus, methods for lead optimization via protein engineering could be critical to bringing RMT-based drug delivery to the forefront. There exist well-characterized methods for protein engineering and fine-tuning of properties such as binding and stability, but they typically rely on access to purified and soluble antigen. However, RMT targets are of course membrane proteins and can be difficult to purify in active forms and in useful amounts. Recently there have been technical advances allowing for antibody engineering using cell and virus-based lysates as membrane protein sources for antibody engineering. Our laboratory has put forth substantial effort to adapt the yeast display immunoprecipitation procedure such that antibodies can be engineered using cell lysates directly as membrane protein sources [41]. In addition, Genentech researchers have reported an analogous approach utilizing detergent-solubilized baculovirus-based membrane protein preparations along with antibody phage display [101]. It is our view that protein engineering will be necessary to realize the real potential of the lead RMT molecules discussed throughout this review. In conclusion, there has been a substantial amount of progress in applying combinatorial screening technology to the BBB targeting and transport problem; however, many challenges and opportunities for technical innovation remain.

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ABBREVIATIONS

AAV	Adeno-associated virus
BBB	Blood-brain barrier
BMSPA	BBB membrane protein-specific polyclonal antibody
BSAT1	BBB specific anion transporter type 1

CNS	Central nervous system
CP	Choroid plexus
LINCL	Late infantile neuronal ceroid lipofuscinosis
LRP	Low density lipoprotein receptor-related protein
MPS VII	Mucopolysaccharidosis type VII
MS/MS	Tandem mass spectrometry
NCAM	Neural cell adhesion molecule
RMT	Receptor mediated transcytosis
SAGE	Serial analysis of gene expression
sdAbs	single domain antibodies
SSH	Suppression subtractive hybridization
Tf	Transferrin
TfR	Transferrin receptor
VH	Antibody variable heavy chain
VL	Antibody variable light chain
scFv	Single-chain antibody
YDIP	Yeast surface display immunoprecipitation

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Figure 1.

Transcytosis Pathway into the Brain. The cartoon represents a simplified view of how a peptide or antibody targeted to an endogenous transcytosing receptor would gain entry into the brain. 1. Antibody or peptide binding: The antibody or peptide binds to its RMT target, preferably at a site distal to the endogenous ligand binding site. 2. Ligand binding and receptor clustering: Here the endogenous ligand binds to two receptors, which is characteristic of the transferrin receptor. Generally speaking if the internalization is ligand binding dependent, the receptors will cluster prior to internalization. However, it is also possible that steps one and two could happen in the reverse order. 3. Endocytosis initiation: invaginations start to form often mediated by either clathrin or calveolin. 4. Vesicles are trafficking through the cell: This part of the process has not been fully elucidated, but sorting takes place in the early endosome before the cargo is trafficked across to the basolateral membrane. 5. Exocytosis of payload: Vesicle merges with basolateral membrane and the payload is released.

Figure 2.

Basic combinatorial screening methodology for BBB transporter discovery. 1. Creation or selection of diverse library comprising DNA encoding the proteins to be displayed. 2. Selection of display platform and display of DNA-encoded library. Subtraction can be performed at this step to help screen for targeting ligands having some brain specificity. 3. Presentation of complex antigen pool containing unknown RMT systems. 4. Wash: Removes unbound library molecules and loose binders, washing stringency can be adjusted to fit the needs of the screen. Moreover, the screen can be designed to select those peptides or antibodies that bind, internalize, or transcytose. 5. Library Recovery: The clones that remain after the wash step are recovered by scaffold-appropriate methods (e.g. bacterial infection, or PCR amplification). 6. Amplification: The recovered clones are amplified for the next round of screening. Steps 1-6 can be repeated until the pool has been enriched for BBB-binding peptides and antibodies. 7. Validation and Characterization of Targeting Reagents: The recovered peptide or antibody clones are tested for the presence of desired phenotype. 8. Antigen Identification: The RMT target bound by peptide or antibodies recovered in the screen is identified. The entire procedure could also be performed substituting the cell-based substrate in step 3 with a known purified RMT receptor that has been identified as a drug delivery target by other methods such as genomics or proteomics.

A) Antibody Library Formats

B) Display Platforms

Figure 3.

A) Antibody Library formats: sdAbs consist of the variable heavy chain (VH) only. The scFv format consists of the VH region connected to the variable light chain (VL) by a polypeptide linker. Fabs consist of the full light chain, including the constant light region (CL), as well as the VH region fused with the first constant heavy region (CH1). Usually it is expressed as two chains, but single-chain versions have been used. The IgG is a full-length antibody, which includes the Fc region and two binding sites. B) Display Scaffolds: In phage display, the library is typically displayed on the pIII protein (depicted by red circle). On each phage particle, 0-5 copies of the library protein can be displayed. In yeast display, the library is fused to the Aga2p mating protein which gets trafficked to the cell wall for display. It has 30,000-100,000 copies of the library protein on its surface. In AAV display, the library format is usually limited to peptides due to capsid protein packing constraints, although limited scFv display has been performed [102]. As an alternative, the coat proteins themselves can be mutagenized to offer different cell tropisms.

Figure 4.

Schematic of expression cloning method. (1) Transfect the host cells with the plasmid-based cDNA library, (2) Express the antigens on the host cell surface and label the cells with peptide or antibody generated in the combinatorial screen, (3) and (4) Isolate the positively labeled cells by fluorescence activated cell sorting (FACS), (5) Extract and amplify the plasmid, (6) Transform the enriched cDNA pool for next round screening, (7) Sequence the selected plasmid encoding the RMT system targeted by the antibody or peptide.

Table 1

Studies employing combinatorial screening methods to isolate BBB targeting peptides, antibodies and viruses.

Library Scaffold	Platform	Substrate Type (Administration)	Validation	Reference
Synthetic Cyclic 7mer	Filamentous phage	Mouse brain, <i>in vivo</i> (intravenous)	The isolated clone mimics iron when binding to Tf. It was used for imaging/gene delivery to brain tumors <i>in vivo</i> .	Staquicini et al., 2011 [13]
Human derived scFv, nonimmune	Yeast	RBE4 Cell line	Many scFv were shown to bind and some to internalize into RBE4 cells. The scFv also showed <i>in vivo</i> binding and were characterized by YDIP.	Wang et al., 2007 [40]
Human derived scFv, nonimmune	Yeast	RBE4 Cell Lysate	Many scFv were shown to bind specifically to RBE4 plasma membrane proteins. Some limited antigen characterization was performed via yeast display immune precipitation.	Cho and Shusta, 2010 [51]
Camelid derived sdAbs, nonimmune	Filamentous phage	Human primary endothelial cells	The isolated clones were shown to transcytose a human EC based transwell BBB model, and home to the mouse brain <i>in vivo</i> .	Muruganandam et al., 2001 [26]
Synthetic 12mer	Filamentous phage	Mouse brain, <i>in vivo</i> (intravenous)	TGN coated nanoparticles internalized into the bEnd.3 mouse EC line and, <i>in vivo</i> imaging indicated that the nanoparticles localized to the mouse brain.	Li et al. 2011 [56]
Synthetic Cyclic 7mer	Filamentous phage	Mouse brain, <i>in vivo</i> (intravenous)	The isolated clone, when produced as a soluble peptide, localized to mouse brain, as characterized by <i>in vivo</i> imaging.	Li et al. 2012 [57]
Synthetic 15mer	Filamentous phage	Mouse brain, <i>in vivo</i> (perfused)	The isolated clones showed brain accumulation <i>in vivo</i> after perfusion. The clones also showed binding to the hCMEC/D3 cell line.	Van Rooy et al., 2010 [29]
Synthetic Cyclic 7mer	Filamentous phage	Rat brain, <i>in vivo</i> (nasal passage)	One clone was shown to enter the rat brain via nasal passage at higher levels than controls.	Wan et al., 2009 [62]
Synthetic Cyclic 7mer	Filamentous phage	Mouse choroid plexus explant, <i>in situ</i>	Three clones were shown to internalize into the CP epithelia, both <i>in vivo</i> through i.c.v. injection and <i>in vitro</i> .	Gonzalez et al., 2011 [63]
Synthetic 7mer	Filamentous phage	Normal mouse brain, and diseased state mouse brains, <i>in vivo</i> (intravenous)	Peptide tags were inserted into AAV coat proteins and the decorated AAV showed brain endothelial cell transduction <i>in vivo</i> in normal and diseased model mouse. The decorated AAV particles improved disease symptoms when harboring DNA coding for missing enzymes.	Chen et al., 2009 [28]

Library Scaffold	Platform	Substrate Type (Administration)	Validation	Reference
Shuffled AAV coat proteins	AAV capsid	Seizure compromised rat brain, <i>in vivo</i> (intravenous)	The isolated AAV clones were shown to transduce neurons around seizure compromised portions of rat brain, but not normal tissue.	Gray et al., 2010 [47]
Synthetic Cyclic 7mer	T7 Phage	Rat ischemic stroke model, <i>in vivo</i> (intravenous)	The isolated peptide was shown to bind selectively to stroke damaged tissue in rat brain, and to preferentially target apoptotic neuronal cells.	Hong et al., 2008 [65]
Camelid derived sdAbs, immune	Filamentous phage	Mouse glioblastoma xenograft, <i>in vivo</i> (intravenous)	The isolated clones were shown to bind brain tumor vasculature preferentially to normal brain vasculature.	Roodink et al., 2010 [59]