Targeting Central Nervous System pathologies with nanomedicines

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Abstract

One of the major challenges in drug development is the delivery of therapeutics to the central nervous system (CNS). The blood-brain barrier (BBB), which modulates the passage of molecules from the CNS, presents a formidable obstacle that limits brain uptake of therapeutics and, therefore, impedes the treatment of multiple neurological pathologies.

Targeted nanocarriers present an excellent opportunity for drug delivery into the brain leveraging on endogenous receptors to transport therapeutics across the BBB endothelium. Receptor mediated transport endows multiple benefits over other, conventional delivery methods such as the transient permeabilization of the BBB or the direct depositioning of intracranial depots. Herein, different strategies for nanocarrier targeting to the CNS are discussed, highlighting the challenges and recent developments.

1. Introduction

The field of neuroscience has made a great progress over recent years, which led to an improved understanding of central nervous system (CNS) disorders. Yet the progress in the development of successful strategies for treating these disorders is still lagging behind. The CNS, consisting of the brain and spinal cord, has a protective barrier in the form of the blood brain barrier (BBB), which limits the entrance of many therapeutics in different types of CNS disorders. This is one of the reasons why CNS

therapeutics take longer to develop and have a lower clinical approval success rate in comparison to other drugs (according to the Tufts Center for the Study of Drug Development http://csdd.tufts.edu). The BBB acts as a highly selective and restrictive barrier in order to protect the CNS from the passage of neurotoxins, invading organisms and to regulate the movement of essential nutrients between the circulation and the brain parenchyma. This major challenge led to an entire field of research dedicated to overcoming the BBB and efficiently delivers therapeutic agents to the brain. The BBB is formed by endothelial cells joint together by tight junctions that line cerebral vessels [1, 2, 3] (Figure 1). This causes most molecular substances to pass through a transcellular route across the BBB rather than a paracellular route through the junctions, as in most endothelial barriers [4, 5].

The organization of the BBB components and the function of the BBB can be altered under pathological condition such as brain cancer, multiple sclerosis, dementia, epilepsy and more. In the case of brain malignancies, a blood-brain-tumor barrier (BBTB) can be formed with the progression and deterioration of the disease. The BBTB contain existing and newly formed blood vessels that contribute on one hand to the delivery of nutrients and oxygen to the tumor and facilitate glioma cell migration to other parts of the brain and on the other hand block the entrance of therapeutic agents to the brain. Many efforts have been made to overcome BBB or BBTB separately. However, in most cases, BBB and BBTB exist simultaneously when glioma is diagnosed, thus most studies does not distinguish between them and target both of them. However, dual targeting approaches both BBB and BBTB have also been utilized [6].

1.1 Main routes for molecular traffic across the BBB

The movement across the BBB can occur in several cellular pathways (Figure 1). Transcellular passive diffusion involves the movement of solutes across membranes along their concentration gradient without consuming energy or involving a carrier protein. Lipid solubility, polarity, molecular size, concentration in the blood, and surface area available for diffusion can influence the ability of a certain substance to passively diffuse through the lipid membrane of the endothelial cells composing the BBB [5, 7, 8]. Small gaseous molecules (e.g O_2, CO_2)[1], small lipophilic agent (e.g ethanol)[1] and drugs such as the opioids family (e.g morphine, heroin)[9] or antidepressants [10] can passively diffuse through the lipid membranes. Other substances, such as small or large hydrophilic molecules, relay on transcytosis, which can be mediated either by

transport proteins, receptors or through adsorption. A number of specific transport and enzyme systems, which are presented on the apical and the basolateral endothelial membranes, can regulate essential nutrients traffic across the endothelial cells from the blood into the brain parenchyma. The complexity of the restrictive passage through the BBB should be taken into account in drug delivery strategies to the CNS. The designed drug construct should target one of the active transcytosis pathways in order to reach the brain parenchyma. The first active transport is the transporter-mediated transcytosis pathway. This pathway is a substrate selective pathway, thus the suggested drug should either mimic the endogenous substrate or have the natural substrate bound to the drug conjugate [11]. The endothelial cells incorporate either influx transport carriers for glucose (e.g GLUT-1 glucose carrier) [12, 13, 14], amino acids (e.g L1 amino acid transporter(LAT1)), glutathione [15], nucleosides, and other substances or efflux transporters (e.g. P-glycoprotein, Multidrug resistance-associated protein (MRP1/4/5)) [16, 17], which are energy-dependent. Hydrophilic macromolecules and certain peptides can cross through the BBB by endocytic mechanisms involving a receptor in a process called receptor-mediated transcytosis (RMT) [18] and by a less specific process called adsorptive-mediated transcytosis (AMT) [19, 20]. These processes include a vesicle-mediated transfer of substances across the cell by endocytosis at the apical membrane and the release of the content to be exocytosedat the basolateral membrane [21]. Several receptors that are expressed on the endothelium of the brain can be used in devising delivery strategies. Among them are the transferrin receptor (TfR) [22, 23, 24], low-density lipoprotein receptor (LDLR) [25, 26], insulin receptor (IR) [27], and others as detailed below. Compared to the RMT pathway, which requires as a first step specific binding of the ligand to a membrane receptor followed by internalization, the AMT pathway involves a nonspecific binding of the ligand to membrane surface charges as a first step, then followed by internalization. The interaction of a desired cationic protein or cell penetrating peptide (CPP) is based on electrostatic interactions between the positively charged substance and the negatively charged membrane of the endothelial cells [19, 20]. The degree of transcytosis across the brain endothelial cells is to a lesser extent compared to the peripheral endothelium, which makes drug delivery across the BBB even more challenging [1]. Nevertheless, the endothelium of brain arterioles and venules were reported to be leakier and subjected to greater modulation [1, 28]. This is a great advantage for delivering drugs across the brain once passing the less penetrable brain capillary endothelium.

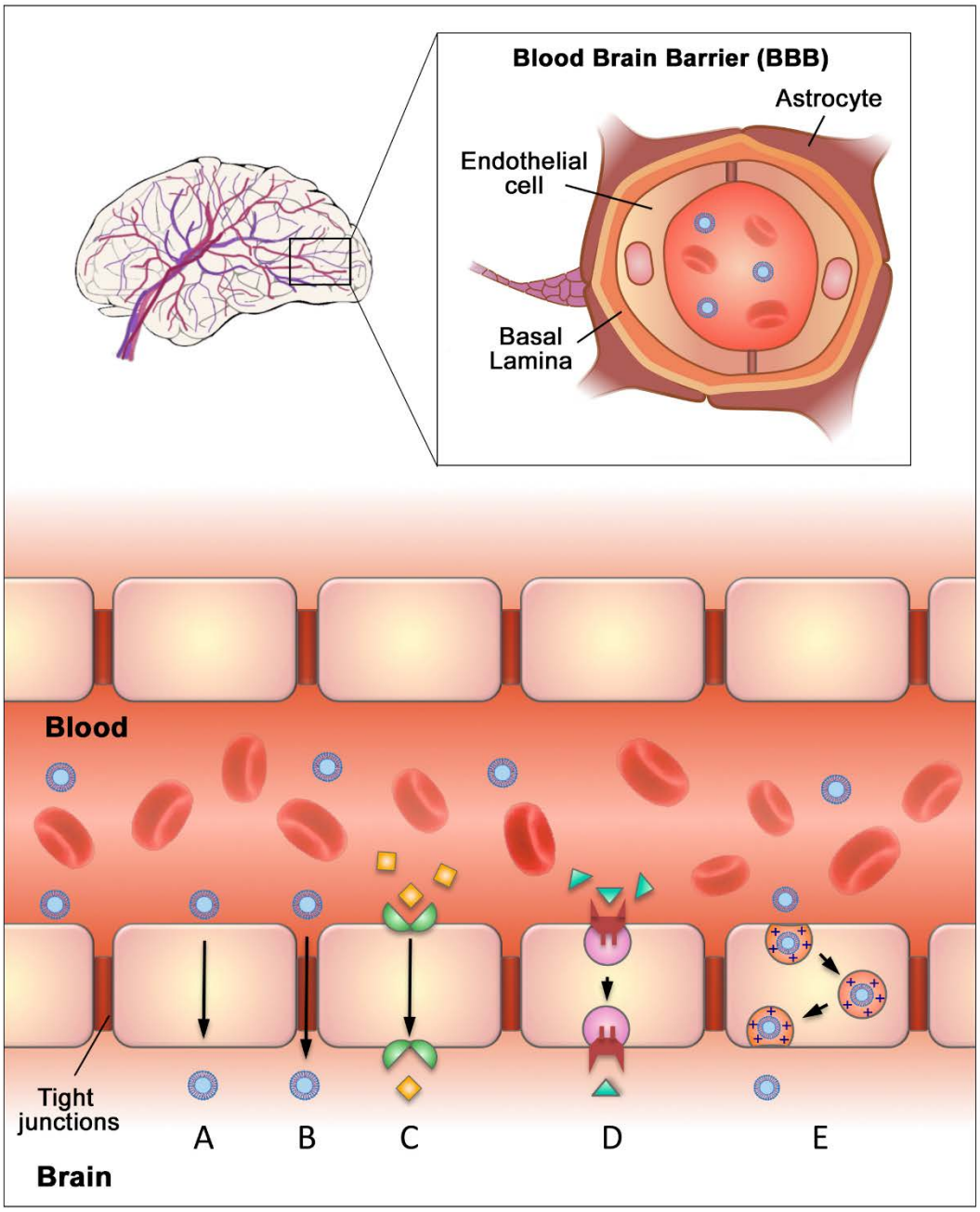

Figure 1: Schematic representative of the Blood Brain Barrier (BBB) and physiological pathways that enable nanoparticle based drug delivery across the BBB. (a) Paracellular pathway; (b)Transcellular pathway; (c) Transporter-mediated transcytosis; (d) Receptor-mediated transcytosis; (e) Adsorptive- mediated transcytosis.

1.2 Delivery routes of therapeutics to the CNS

Although many drugs have therapeutic potential for CNS disorder, only few of them have been clinically used due to limitations posed by the BBB. The delivery routes for the CNS can be divided into two main approaches: invasive and non-invasive. The invasive approach mostly relies on surgical intervention for the administration of the therapeutic agent directly into the brain, hence bypassing the BBB. This intracranial drug delivery includes the methods of intracerebral implementation [29, 30, 31], intracerebroventricular infusion [32], interstitial delivery [32] and convection-enhanced diffusion (CED) [33]. These delivery strategies involve administration of the therapeutic agent by either implanting drug-releasing depots into the brain parenchyma, direct injection into the brain or catheters stereotactically placed through cranial burr holes. This invasive approach for local delivery of drugs to an intracranial target can achieve sustained drug concentrations and is considered to be the most appealing method for the treatment of brain tumors. However, this strategy has certain disadvantages, such as surgery complications, CNS infection, catheter obstruction, potential high intracranial pressures, local toxicity, and inadequate drug distribution.

The noninvasive approach can be achieved by systemic delivery of the therapeutic agents capable of crossing the BBB or by alternative pathways which directly bypass the BBB such as intranasal administration. It is known that the olfactory and trigeminal nerves create a pathway connecting the nasal cavity and the brain thus providing potential routes for noninvasive administration of therapeutics to the CNS [34, 35]. This pathway enables a quick delivery of drugs to the CNS within minutes, especially drugs with lower molecular weight and higher lipophilicity. However, the disadvantage of the use of this method is the concentration that can be achieved in different regions of the brain and spinal cord. High molecular weight drugs tend to be less efficient in this drug delivery method. Finally, systemic administration can be achieved by intravenous (IV) administration. By IV injection, the therapeutic agents encounter the BBB and in order to penetrate the brain should be able either diffuse passively or use the active pathways discussed earlier such as the transport proteins, RMT or AMT. The existence of abundant transporters and receptors on the apical membrane of the BBB and their unique characteristics offer substantial potential for drug development and will be discussed further on.

Nowadays, different methods for disrupting the BBB are being explored in order to enhance the delivery of different therapeutic agents. One such method is the magnetic resonance imaging-guided focus ultrasound (MRI-gFUS) [36]. In this method, microbubbles are injected systemically and under the guidance of MRI, a certain targeted area in the brain is being stimulated by the US waves, causing a disruption of

the BBB. The microbubbles can be loaded with the desired drug, thus can be used as a drug carriers for targeted delivery [37]. Another approach is to inject empty microbubbles in order to cause the temporary breaching of the BBB and then injection of the therapeutic agent [38, 39, 40]. Other ways to promote the temporary disruption of the BBB is to use agents such as mannitol [41, 42] and bradykinin [43, 44].

1.3 Nanoparticle based drug delivery to the CNS

In order to overcome the obstacles mentioned above, many attempts to develop therapeutic agents have been made. This includes viral vectors, nanoparticles (NP), cell penetrating peptides (CPPs) and many more. Viral vectors have become a valuable tool for therapeutic gene delivery to a specific site. A number of different viruses have been studied as vectors for gene CNS delivery. These include lentivirus [45], retrovirus [46], recombinant adeno-associated virus [47] and herpes simplex virus [48]. Although the use of viral vectors demonstrated satisfactory efficiency for CNS delivery, there are several disadvantages that should be considered when approaching this delivery method. These limitations include unwanted immune response, changes in the properties of delivered virus due to endogenous recombination, and mutagenic behavior leading to oncogenesis.

On the other hand, over the past decades, different types of nanoparticles (NPs) have emerged as potential drug delivery vehicles due to the fact that they can be easily tailored to achieve both controlled drug release and tissue specificity [49, 50, 51]. NPs are small-scaled drug carriers that can vary in shape, size, surface properties and mechanical stiffness depending on their composition which can be based on different materials such as lipids, polymers, proteins, or inorganic compounds [52, 53]. NPs serve as carriers for a variety of therapeutic molecules: from nucleic acid to proteins, small molecules and chemotherapy drugs, and combinations of the aforementioned agents. NPs are characterized in the ability to protect the payload from degradation, increase the plasma concentration half-life, reduce toxicity, and release the payload in a controlled release manner, hence enabling a wide therapeutic window [49, 50]. In addition, NPs can promote delivery of their cargo directly to specific cells and are therefore can be used for precise tumor targeting.

The physicochemical characteristics of NPs have been shown to be crucial for determining their fate and performance following administration. Several parameters were examined including nanoparticle size, shape, stiffness, surface charge, composition, and aggregation. Size has a significant effect on blood circulation time as an optimal vesicle should be large enough to avoid renal clearance but small enough to avoid clearance via the mononuclear phagocytic system [54]. The size of NPs

should be also adjusted in accordance to the route of administration and delivery purposes. The shape and curvature of NPs can also affect circulation half life and immune response as oblate-shaped NPs were shown have a lower macrophage uptake in comparison to spherical nanoparticles and, therefore, longer circulation time and different biodistribution [55]. NP softness is another important factor that has recently been shown to be a key parameter in modulating the behavior of nanoparticles. It has been shown that for polymeric nanoconstructs, regardless of the size and shape, softer NPs were significantly better in avoiding uptake by bone-marrow-derived monocytes in comparison to rigid ones [53]. Moreover, softer NPs, have been shown to enhance tumor vasculature targeting [52]. Surface features of LNPs have also been thoroughly investigated. Positive charge has been shown to result in better uptake of by cells in comparison to uncharged or negatively charged NPs [56]. However, positively charged NPs have toxic effects including induction of pro-inflammatory response [57]. Due to the importance of surface characteristics, surface functionalization has been widely used either for elongation of circulation time by hydrophilic polymers such as PEG and Hyaluronan; or for specific cell targeting by ligands such as antibodies, peptides, and aptamers.

The physicochemical properties of NP also influence their passage across the BBB, naturally, long circulation will promote BBB passage but there are additional factors. Size is an important factor as small particles have been shown to achieve better BBB penetration [58]. Indeed, most studies detailed below utilize NP smaller than 200nm. Nevertheless it should be noted that filamentous phages used for BBB penetrating peptides in-vivo are 900 nm in length and have a diameter of 6.5 nm. Shape and surface charge have also been studied yet literature report have not been consistent [59].

The complexity of the BBB makes CNS drug delivery a tremendous challenge, but also provides many unique opportunities for drug delivery. The conjugation or adsorption onto the NP surface of moieties that could interact with the BBB and facilitate transcytosis are examples of promising approaches to drug delivery. Herein, we will focus on non-viral targeted drug delivery systems, which have demonstrated the ability to cross the BBB and deliver therapeutic payloads *in-vivo*.

2. Specific targeting

Large hydrophilic molecules can cross the BBB by either RMT or by AMT [1, 60] (Figure 1). RMT possess several advantages in comparison to AMT and untargeted systems. As mentioned passive delivery of untargeted systems mostly enable the passage of small hydrophilic compounds. In addition, both untargeted and AMT based

systems lack specificity. The AMT strategy possess additional limitations such as toxicity and immunogenicity attributed to the positively charged CPPs used as detailed below (PMID 18726697).

From a clinical standpoint there are many advantages for developing NPs, which utilize the RMT approach for crossing the BBB as the entry mechanism is known and therefore such systems are likely to receive faster regulatory approval. Therefore, in this review we will focus on targeted NPs that have a known receptor/transporter.

There are many sources for specific ligands among which are endogenous neurotropic biomolecules, pathogen/ toxin derived proteins, and peptides and phage display bio-panning as detailed below.

2.1 The ultimate receptor/transporter

Upon examining the receptor/transporter for CNS drug delivery, there are several required attributes. The first attribute is specificity: the receptor should be exclusively expressed (or at least expressed in higher amounts in comparison to other tissues) in the apical side of brain vasculature. The second and obvious requirement is the capacity to facilitate transcytosis. High turnover is another requirement and as other requirements it is common for all receptors utilized for active drug targeting. Another important attribute is the physiological role of the transporter that should not be easily altered [61].

Due to high expression on the BBB and their capacity to facilitate transcytosis, several endogenous transporters and receptors have been used for BBB targeting among them are transferrin receptor 1 (TfR1), glucose, GSH, low-density lipoproteins (LDLRs), insulin receptor (IR), and leptin.

2.2 Transferrin receptors

The concept of utilizing the transferrin receptor for brain drug delivery has been proposed already in 1984 by Jefferies et al. [62, 63] and has been extensively studied as a potential brain drug delivery target [64]. The rationale behind using TfR is clear as it is responsible for the transport of transferrin to the brain parenchyma in order to maintain the iron homeostasis. In addition, TfRs are highly expressed on brain capillary endothelial, but not on endothelial cells elsewhere in the body [64].

Several strategies have been used for TfR targeting among them are antibodies, peptides and transferrin as the natural ligand for the delivery of a wide array of nanoparticles and therapeutics [64]. Interestingly, the affinity of the targeting moiety towards the receptor has been shown to be highly important as low affinity antibodies were shown to be superior over high affinity antibodies as far as brain uptake upon

intravenous IV administration to mice [65]. This is due to the fact that Anti-TfR antibodies that bind with high affinity to TfR remain associated with the BBB, whereas lower-affinity anti-TfR antibody variants are released from the BBB into the brain and therefore show a broad distribution as demonstrated by Mark S. Dennis and colleagues [65].

Patrick Couvreur and colleagues have developed chitosan−PEG nanoparticles functionalized with the monoclonal antibody OX26 against TfR for brain delivery of the caspase-3 peptide inhibitor [66]. The authors have shown brain localization following IV administration of the NPs. In a following studies the authors have shown that by using the TfR targeting approach they can provide neuroprotection upon systemic administration [67, 68]. In a recent study, the authors also address an important issue in the development of brain targeted DDS which is the evaluation of the underlying mechanisms for enhanced cellular entry [69]. The same group also developed squalenoyl adenosine nanoparticles, though untargeted these particles managed to provide neuroprotection after stroke and spinal cord injury [70].

2.3 Low-density lipoprotein receptors (LDLRs) and low-density lipoprotein receptor-related proteins (LRPs)

Like TfR, LDLRs and low-density lipoprotein receptor-related proteins 1 (LRP1) and 2 (LRP2) have been also widely studied as potential receptors for delivery across the BBB due to their high expression levels in brain capillary endothelial cells. LRP1, for example, have been shown to be overexpressed in the brain [61, 71, 72]. In addition, the RMT capacity of LDLRs is potentially higher than that of TfRs [72, 73].

LDLR targeting can be achieved directly by coating the NP with antibodies, specific ligands or peptides derived from ApoB, ApoE and Angiopep-2 or indirectly by nanoparticle surface modification that leads to the adsorption of apolipoproteins [74, 75, 76].

One of the most advanced BBB delivery methodology is based on targeting with Angiopep-2, a peptide identified through sequence alignment screening with other human proteins having a Kunitz domain, which interacts with LRP1 [61, 77, 78]. Angiopep-2 has shown a high rate of transcytosis and brain uptake [61] and rapidly matured to phase II clinical trials with a paclitaxel conjugate for the treatment of breast cancer (NCT01967810) [79] and high-grade glioma in combination with bevacizumab (NCT01480583). Angiopep-2, has also been used for the delivery of a wide array of NPs encapsulating either small molecules, proteins or nucleic acid-based therapeutics into the CNS [80]. A recent work reports dual targeting immunoliposomes encapsulating TMZ using Angiopep-2 and CD133 antibody for glioblastoma stem cells

[81]. The authors report a significant reduction of tumor size following IV administration in addition to increased lifespan. However, the authors did not compare the dual targeted liposomes to liposomes only targeted with Angiopep-2 and therefore the added value of the addition of CD133 is not clear.

2.4 Glutathione (GSH) transporters

Another targeting moiety that has progressed to to clinical trials is GSH. GSH transporters is a great example of utilizing the selective transport of nutrients to facilitate drug delivery into the brain. GSH is an endogenous tripeptide that possesses antioxidant-like properties and is therefore highly important for the detoxification of intracellular metabolites [82].

2-BBB's G-Technology® developed two GSH targeted liposomal products currently in clinical trials: 2B3-101 (glutathione PEGylated liposomal doxorubicin) and 2B3-201 (glutathione PEGylated liposomal methylprednisolone). 2B3-101, developed for patients suffering from multiple brain cancer indications, with an initial focus on patients with brain metastases of breast cancer and patients with glioma has completed a Phase I/IIa clinical trial (NCT01386580). 2B3-201, developed for patients suffering from acute and chronic neuro-inflammatory diseases, with an initial focus on patients with acute MS relapses completed phase I study (NCT02048358). In preclinical evaluation, 2B3-201 has shown a favorable pharmacokinetic profile and optimal distribution to the brain compared to the free drug [82, 83].

The technology has also been used in preclinical studies for the delivery of additional therapeutics such as small molecules, peptides and antibodies [83, 84, 85, 86].

3. Going beyond the obvious

3.1 Pathogen and venom derived targeting moieties

Despite the large amount of literature evidence for the well-known RMT targets described above, including TfR, LDLR and insulin receptor (reviewed elsewhere [72]), these receptors are expressed in multiple tissues and are not brain or BBB specific. Therefore, these RMT targets would be suitable for the treatment of deficiency syndromes but not for cases in which specific delivery is required such in the case of tumors, neurodegenerative and certain neurodevelopmental disorders. In addition, all of these receptors are characterized with a relatively low RMT capacity and therefore low levels of brain uptake (<2% in most systems). Incidentally, it should be explained that accurate quantification is another hurdle for BBB delivery. Thus, the identification of novel BBB RMT ligands with better tissue specificity and improved RMT capacity is yet an unmet need. There are increasing efforts for identification of such targets both

through screening of peptide libraries using several approaches and by exploring pathogen derived targets as detailed below.

The rationale of utilizing pathogen derived ligands is very intriguing as these targeting moieties are a product of long evolutionary processes for achieving CNS entry and therefore highly verified. Thus, inquiring of pathogen (viral or bacterial) and venom derived targets supplies a new pool of previously unexplored targets. Particularly interesting is the acetylcholine receptor (nAchR) which is targeted by the Rabies virus, plant extract alkaloid arrow poisons and snake venom components such as three-finger toxins (Table 1 & Figure 1). Nevertheless, it should be noted that pathogen and venom derived proteins are both toxic and highly immunogenic in their complete form. Therefore, the specific fraction sufficient for targeting without inducing unwanted effects should be isolated. The general concept of using pathogen inspired systems was reviewed elsewhere [87, 88]. Here, we will focus on systems that matured to *in-vivo* delivery of therapeutics.

As described in Figure 2, pathogens do not only utilize CNS receptors for cell entry but also exploit several cellular mechanisms for CNS penetration. For example, several pathogens utilize intracellular transport, a mechanism essential for the distribution of neuronal organelles and proteins. The retrograde transport facilitated by the cytoplasmic dynein motor enables transfer of cargo from the nerve terminus to the cell body. The use of motor based axonal transport is a key mechanism for viral spread across the CNS especially when taking into account axon length which makes relying on passive diffusion irrelevant [89]. The use of motor based axonal transport following entry at peripheral nerve endings have been documented for several neurotropic viruses, bacteria and toxins including the Rabies, Poliovirus, Canine adenovirus type 2 and Tetanus toxin [89]. Therefore, it is possible that NPs conjugated with viral proteins will also be able to exploit the motor based axonal transport for CNS spreading.

The specific mechanisms of viral entry have also been studied and certain molecules on neuromuscular junctions and sensory-nerve endings can serve as receptors. For example, Poliovirus binds CD155, several adenoviruses bind the coxsackievirus and adenovirus receptor (CAR) and Rabies virus binds p75, nicotinic acetylcholine receptor (nAChR) and neural cell adhesion molecule (NCAM) [89].

3.1.1 Rabies virus glycoprotein (RVG)

The concept of harnessing RVG for the purpose of drug delivery was reported by Priti Kumar and colleagues in 2007 [90]. These researchers were the first to raise the hypothesis that the strategy of BBB crossing of neurotropic viruses can be utilized for

siRNA delivery to the brain. The authors chose Rabies probably due to the fact it has a known neurotropism and in addition a lot is known regarding its CNS entry mechanism and specific receptors (Figure 2). The peptide used for targeting in this study, RVG29, was first discovered upon studying the CNS tropism of rabies [90, 91, 92]. In these studies it was found that a 29 residue fragment of RVG (RVG-29) was able to completely inhibit the binding of snake-venom toxin α-bungarotoxin (BTX) to AchR [90, 91]. To enable siRNA binding, Kumar and colleagues synthesized a chimeric peptide by adding the positively charged nonamer arginine residues at the carboxy terminus of RVG-29. The authors have shown that the RVG-siRNA conjugate managed to reach the brain following IV administration and even promote a therapeutic effect by affording robust protection against fatal viral encephalitis in mice.

Ever since, RVG was used for the delivery of multiple therapeutics and a wide array of nanoparticles and even exosomes, as summarized in Table 1. The different strategies of RVG targeting have been recently reviewed [93].

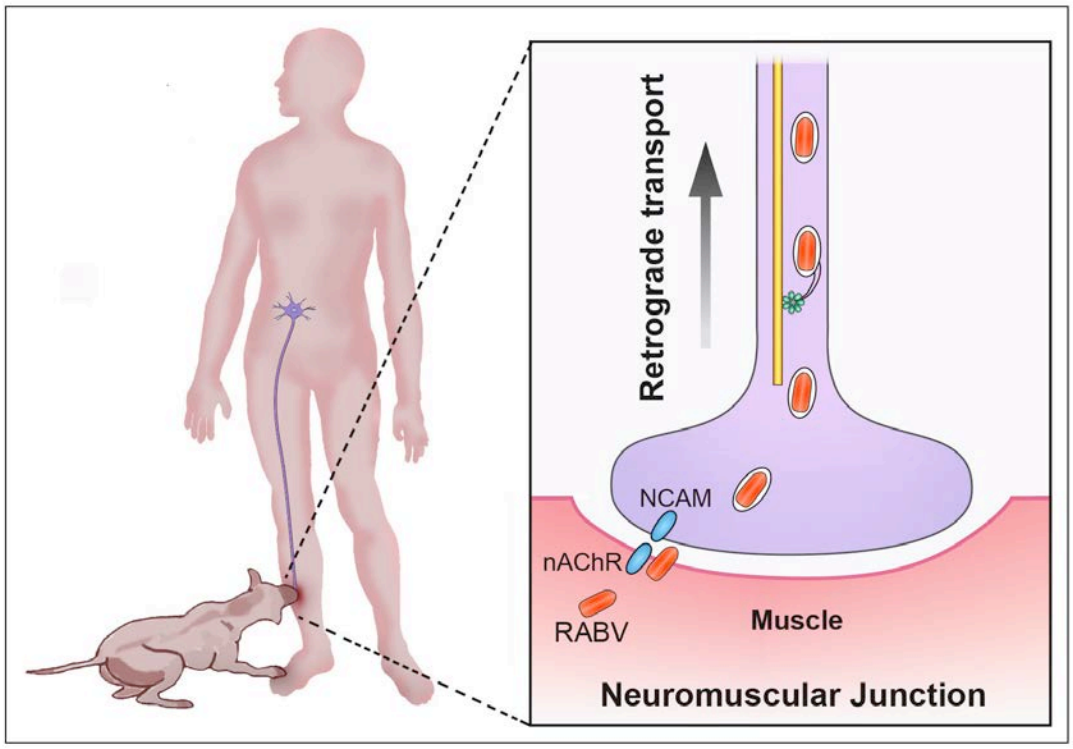

Figure 2: Rabies virus axonal transport. The long axonal transport to the CNS begins in the periphery. According to the classical pathway, the rabies virus first infects muscle cells upon binding to the acetylcholine receptor. From the muscle, the virus spread into the neuromuscular junction and enters neurons via NCAM or p75. In the neuron, the Rabies virus utilizes the cells' retrograde transport to travel to the CNS.

Nevertheless, upon using NPs for delivery, the biodistribution is less favorable in comparison to siRNA conjugates reported by Kumar and colleagues as most of the injected dose would eventually end up in mononuclear phagocytic system (MPS) related organs. Therefore, improving NP biodistribution, which is a general challenge for all drug delivery purposes is critical when it comes to CNS delivery.

3.1.2 Chlorotoxin (CTX)

Chlorotoxin (CTX) is a 36 residue peptide derived from the venom of the Israeli scorpion Leiurus quinquestriatus [94]. This peptide has been used in multiple CNS delivery systems and even matured to clinical trials due to its ability to preferentially bind to brain tumor cells and high stability derived from its 8 cysteines that form 4 disulfide bonds [94, 95, 96]. The preferential binding of malignant tissue may be related to CTX's specific interaction with metalloprotease-2 (MMP-2) which is overexpressed in brain tumors. Additional advantages for CTX from a drug delivery standpoint are its lack of toxicity and immunogenicity and demonstrated ability to enter the brain upon IV administration, suggesting that it might be able to cross the BBB directly [94]. CTX has also been shown to possess antiangiogenic activity, additional advantage for cancer therapy [97].

CTX's preferential binding to tumor cells has been harnessed for both radiotherapy and for the development of imaging agent to help tumor visualization during surgical resection [94, 95]. TM-601, an iodine 131 radioconjugate of the synthetic CTX, is currently evaluated in phase II clinical trials for the evaluation of safety and efficacy in high-grade gliomas (NCT00114309, NCT00683761) [98, 99, 100].

CTX has been used for systemic delivery of a wide array of therapeutics such as methotrexate, cisplatin, alisertib, siRNA and DNA and with several types of NPs including liposomes, dendrimers and polymeric NPs, as summarized in Table 1.

Recently, Tamborini M et al. presented a combined approach employing both CTX targeted PLGA nanoparticles and radiation to reach infiltrating tumor niches in GBM [101]. The authors have shown the necessity of the combined approach as the whole brain X-ray irradiation prior to the injection of the targeted NPs enabled enhanced expression of CTX targets (including MMP2) and possible BBB permeabilization that enhanced the amount of targeted NPs in dispersed tumor cells. Another paper that demonstrated inhibition of GBM utilizing CTX targeted NPs was recently published by Han L. et al. [102]. The authors utilized an interesting "autocatalytic" approach to increase brain NP accumulation by encapsulation of BBB modulators in CTX targeted

NPs. This enables the transportation of more NPs by creating a positive feedback loop. Moreover, the authors have shown that preferential accumulation in brain tumors at a concentration of 4.3- and 94.0-fold greater than that in the liver and in brain regions without tumors.

Additional pathogen and venom derived targeting moieties including Clostridium tetani, Vibrio Cholerae, mamba Snake venom and bee venom are also listed in Table 1.

3.2. Phage display screening

Phage display is an effective molecular technique based on a direct linkage between phage phenotype and its encapsulated genotype, which leads to presentation of molecule libraries on the phage surface. This technique is being utilized in studying interactions between a protein and its ligand, receptor binding sites, and in improving the affinity between a certain protein to its binding ligand. Phage display is an efficient method for obtaining specific proteins and peptide that can bind to a certain receptors, thus provides a key tool for identifying novel agents (e.g antibodies, proteins or peptide) that can bind the BBB and facilitate the transport between the blood to brain parenchyma in the RMT pathway. Therefore, phage display libraries represent huge potential for formulating targeted drug delivery platforms [103].

3.2.1 FC5

FC5, a single domain llama antibody, is an interesting and relatively new targeting moiety discovered by Stanimirovic D. et al. [104]. FC5 was isolated via antibody phage display screen performed against human cerebromicrovascular endothelial cells (HCEC).The aim of the screen was to identify novel BBB binding and transmigrating antibodies [104] and indeed FC5 exhibited enhanced passage through the BBB *in-vitro* and *in-vivo*. Despite the fact that the screening was performed without aiming for a particular receptor, the authors later reported that FC5 internalization is probably receptor-mediated process and identified the transmembrane protein TMEM-30A as a possible receptor [105]. Recently, the same group reported on the development of bispecific antibodies in which FC5 was used as a BBB carrier [106, 107]. The authors have shown that bispecific antibodies comprised of FC5 and the antibody antagonist of the metabotropic glutamate receptor-1 (mGluR1) can be detected immunohistochemically in brain regions involved in pain processing after systemic

administration. Thus, this bispecific antibody manages not only to cross the BBB but also to spread across the brain. In addition, the authors demonstrated the antibody's ability to engage central mGluR1 receptors and possess analgesic properties in a rodent model of persistent inflammatory pain following IV administration [106].
FC5 has not been used yet for the delivery of NPs across the BBB and it would be interesting to see whether the capacity to deliver 'cargo' across the BBB and into the brain also applies for larger cargos such as NPs.

3.2.2 Pep TGN

Pep TGN is a 12-residue peptide that was found through *in-vivo* phage display screening, as with FC5, there was no aiming for a specific receptor [108, 109] though the authors do suggest that the mechanism is probably receptor mediated taking into account the selective and active transport across brain endothelial cells. Upon conjugation to PEG-PLGA NPs, the authors showed enhanced brain accumulation and lower liver accumulation in comparison to undecorated NPs. In a following study , the authors report that dual functionalization with both Pep-TGN and QSH (a peptide with good affinity to amyloid plaques) results in a beneficial therapeutic effect in a murine Alzheimer's disease model [110].

4. Learning from pathogens- combination of CPP with targeting approach

As pathogens are equipped with more than just a targeting moiety, delivery of therapeutics in significant amounts probably cannot rely on targeting alone. Therefore, several papers have reported on the combination of both cell-penetrating peptides (CPPs) and a targeting moiety.
CPPs are short positively charged peptides, which can thus cross cell membranes via AMT. CPPs were used for the delivery of multiple cargoes including proteins and nucleic acid-based therapeutics. TAT, a CPP derived from HIV, is one of the first examples for the most frequently used CPP for brain delivery.
Very recently, Xi Yu et al. utilized a novel approach to design protein based nanoparticles for the delivery of therapeutic peptides [111]. The formation of activatable protein nanoparticles (APNPs) is performed via self-assembly of three independent polypeptides based on pairwise coiled-coil dimerization. The formed APNPs are activated to release the encapsulated cargo by locally enriched proteases in the disease microenvironment. The authors demonstrated that APNPs encapsulating the TAT conjugated neuroprotective peptide NR2B9c managed to effectively treat stroke in-vivo, by improved the delivery of Tat-NR2B9c to the ischemic

brain,restoring both infarct volume and neurological function. In this study, APNPs encapsulating TAT conjugated NR2B9c outperformed the free peptides.

TAT was first described by Steven F. Dowdy and colleagues [112] who managed to deliver active proteins to mice brains following intraperitoneal injection. However, the TAT conjugated protein was also delivered to all other tissues. Therefore, delivery that is CPP based only will lack brain selectivity. In addition, due to the fact CPPs are positively charged, their use can induce toxicity and undesired immune activation especially in chronic therapy [113]. Thus, identification of novel and less toxic CPP alternatives is another delivery challenge [114].

Several recent delivery systems report on combining the advantages of CPPs with the selectivity of specific ligands. For example, the combination of Angiopep-2 and TAT was shown to be more efficacious in comparison to Angiopep-2 alone for the delivery of paclitaxel in a murine glioma model [115]. Very recently, the same dual targeting combination was also tested in docetaxel (DTX)-loaded polymeric micelles in a glioma murine model [116]. The authors also reported on the beneficial effect of the combination which was also demonstrated in the improved efficacy and reduced toxicity.

Another combination recently published explored the use of the novel human derived CPP dNP2 in addition to cleavable folic acid (FA) for dual targeting of paclitaxel (PTX) loaded liposome for the treatment of glioma [117]. The authors have shown the benefit of acid cleavable FA for enhanced tumor targeting and glioma growth inhibition as cleavable FA targeted liposomes outperformed the non-cleavable version due to enhanced cell uptake. The same group also utilized a similar approach [118] for enhanced breast cancer and brain metastasis therapy. In this animal model, the dual targeted acid cleavable FA liposomes have shown a greater therapeutic effect in comparison to single targeted or non-cleavable FA targeted liposomes. dNP2 is a novel and interesting human derived CPP which was reported to be less toxic and more potent than currently used CPPs [114, 118].

5. Conclusions

Despite the fact that BBB continues to present a formidable obstacle for the treatment of CNS disorders, a lot of progress has been achieved the recent years with drug delivery to the brain. Many systems utilizing RMT targeting strategies have shown impressive brain delivery in preclinical settings and several even matured to clinical trials.

Nevertheless, many aspects of drug delivery to the brain remain a challenging, especially those related to NP selectivity to the brain and non-specific deposition in MPS associated organs. Additional challenges are technical, such as the accurate quantification of NPs and therapeutics delivered to the brain.

The achievement of drug delivery to the CNS in therapeutically relevant amounts will therefore require a combined effort of multiple disciplines including chemistry, materials science, neurobiology and neuroimaging. This will enable to adjust the chemo-physical NP properties for enhanced stability, improve brain penetrability and identify novel targeting moieties and receptor targets that would enable targeting specific parts of the CNS. Further understanding of the molecular mechanisms behind RMT which differs among the specific receptors and transporters targeted and of BBB bypassing strategies is also required.

Table 1: Pathogen and venom derived CNS targeting moieties

Pathogen type	**Pathogen**	**protein/ peptide (the ligand)**	**Known Receptor/ Transporter / Entry**	**Vehicle**	**Payload**	**Delivery route**	**Reference**
Virus	Rabies Virus	RVG29	nAchR, P75, NCAM	siRNA-complex (electrostatic interaction 9r)	siRNA	intravascular	[90]
		RVG29	nAchR, P75, NCAM	Exosomes	siRNA	intravascular	[119]
		RVG29	nAchR, P75, NCAM	DNA-complex (electrostatic interaction 9r)	shRNA coding plasmid DNA.	intravenous	[120]
		RVG29	nAchR, P75, NCAM	PEI nanocarrier	microRNA	intravenous	[121]

		RVG29	nAchR, P75, NCAM	chitosan-conjugated Pluronic-based nano-carrier	protein	intravenous	[122]
		RVG29	nAchR, P75, NCAM	albumin nanoparticles	small molecule (itraconazole)	intravenous	[123]
		RVG29	nAchR, P75, NCAM	polyamidoamine dendrimers (PAMAM)	pDNA	intravenous	[124]
		RVG29	nAchR, P75, NCAM	Trimethylated Chitosan-PEG particles	siRNA	intravenous	[125]
		RVG29	nAchR, P75, NCAM	Silica-Coated Gold Nanorods	-	intravenous	[126]
		RVG29	nAchR, P75, NCAM	liposome	siRNA	intravenous	[127]
		RVG29	nAchR, P75, NCAM	PLGA nanoparticles	small molecule (camptothecin)	intravenous	[128]
		RVG29	nAchR, P75, NCAM	mPEG- PLGA nanoparticle	Deferoxamine	intravenous	[129]
		RVG29	nAchR, P75, NCAM	liposomes	dopamine derivative BPD	intravenous	[130]

		RDP	nAchR, P75, NCAM	protein conjugate/ fusion protein	protein	intravenous	[131]
		RDP	nAchR, P75, NCAM	protein conjugate/ fusion protein	protein	intravenous	[132]
Bacteria							
	Clostridium tetani	Tetanus toxin fragment C	Peripheral nerve polysialoganglioside s	protein conjugate/ fusion protein	protein	intracerebroventricular (i.c.v.) infusion	[133]
		Tetanus toxin fragment C	Peripheral nerve polysialoganglioside s	protein conjugate/ fusion protein	protein	intramuscular or intrathecal injection	[134, 135, 136]
	Vibrio Cholerae	Cholera toxin B subunit (CTB)	GM1 gangliosid e.	protein conjugate/ fusion protein	protein	Oral Delivery	[137]
		Cholera toxin B subunit (CTB)	GM1 gangliosid e.	protein conjugate/ fusion protein	protein	Intra nasal	[138]
		Zonnula occludens toxin (ZOT) ΔG fragment	bind to a surface receptor and cause TJ opening via intracellula	co administration	small molecules (MTX and paclitaxel)	intracarotid cannula	[139]

			r PKC mediated events				
	Corynebacterium diphteria	Cross-reacting material 197 (CRM197)	Diphteria toxin receptor (DTR)	conjugate	horseradish peroxidase	intravascular bolus injection	[140]
		Cross-reacting material 197 (CRM197)	Diphteria toxin receptor (DTR)	PLGA Nanoparticles	fluorescent probes	intravenous	[141]
		Cross-reacting material 197 (CRM197)	Diphteria toxin receptor (DTR)	PEG-polyethylenimine (PEI)-particles	siRNA	intraperitoneal injection	[142]
Venoms							
Snakes	King Cobra Snake	Hannah toxin	nAchR	poly(ethylene glycol)-poly(lactic acid) micelles (PEG-PLA micelles)	small molecules (paclitaxel)	intracranial	[143]
	Malayan krait (Bungarus	(D) CDX	nAchR	liposomes	doxorubicin	intravenous	[144]

	candidus)						
		(D) CDX	nAchR	PEG–PLA micelles	paclitaxel	intravenous	[145]
		(D) CDX	nAchR	red blood cell membrane-coated nanoparticle	doxorubicin	intravenous	[146]
Scorpion	Scorpion Venom (Leiurus quinquestriatus)	Chlorotoxin (CTX)	Chloride channels, CPP direct passage across the BBB	liposomes	doxorubicin	intravenous	[147]
		Chlorotoxin (CTX)	Chloride channels, CPP direct passage across the BBB	PEG-PLGA NPs	lexiscan	intravenous	[148]
		Chlorotoxin (CTX)	Chloride channels, CPP direct passage across the BBB	Redox-responsive magnetic nanoparticle	O(6)-benzylguanine	convection-enhanced delivery	[148]
		Chlorotoxin (CTX)	Chloride channels, CPP direct passage across the BBB	stable nucleic acid lipid particle (SNALP)	miRNA and small molecule (sunitinib)	intravenous	[149]
		Chlorotoxin (CTX)	Chloride channels, CPP direct	poly(amidoamine) dendrimers	131-I	intravenous	[150]

			passage across the BBB				
Bee	Bee Venom Neurotoxin	Apamin	Presumed-KCa channel	poly(ethylene glycol) (PEG) distearoylphosphatidylethanolamine (DSPE) micelles	curcumin	intravenous	[151]
	Bee Venom Neurotoxin-Apamin fragment	MiniAp-4	Presumed-KCa channel	cyanine 5.5 conjugate	Cy5.5	intravenous	[152]

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