BBB-targeting, protein-based nanomedicines for drug and nucleic acid delivery to the CNS

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Abstract

The increasing incidence of diseases affecting the central nervous system (CNS) demands the urgent development of efficient drugs. While many of these medicines are already available, the Blood Brain Barrier and to a lesser extent, the Blood Spinal Cord Barrier pose physical and biological limitations to their diffusion to reach target tissues. Therefore, efforts are needed not only to address drug development but specially to design suitable vehicles for delivery into the CNS through systemic administration. In the context of the functional and structural versatility of proteins, recent advances in their biological fabrication and a better comprehension of the physiology of the CNS offer a plethora of opportunities for the construction and tailoring of plain nanoconjugates and of more complex nanosized vehicles able to cross these barriers. We revise here how the engineering of functional proteins offer drug delivery tools for specific CNS diseases and more transversally, how proteins can be engineered into smart nanoparticles or 'artificial viruses' to afford therapeutic requirements through alternative administration routes.

Keywords: Nanoparticles; BBB; Protein engineering; Recombinant proteins; Artificial viruses; Drug delivery; Gene therapy

1. Introduction

The maintenance of the central nervous system (CNS) homeostasis is essential for its normal function. The limits of the CNS tissue are established by the astrocytic glia limitans facing the meninges and the blood vessels, and by the ependimocytes of the choroid plexus were the cerebrospinal fluid is produced (Figure 1 A). Astrocyte end-feet wrap the meningeal fibroblasts and the endothelial cells (ECs) of the capillaries, leaving between them the basement membrane. Brain capillaries display a large surface area (~20 m^2 per 1.3 kg brain), and thus possess a predominant role in regulating the brain microenvironment. The blood-brain-barrier (BBB) limits the entry of blood-derived molecules and circulating leukocytes, protecting the CNS from fluctuations in plasma compositions or circulating agents such as neurotransmitters and xenobiotics. It is composed of specialized ECs held together by multiprotein complexes known as tight junctions, astrocytes, pericytes and basement membrane (Abbott et al. 2006; Reese and Karnovsky 1967) (Figure 1 B). CNS ECs display more efficient cell-to-cell tight junctions than other ECs (Wolburg and Lippoldt 2002), rest on a continuous basement membrane and express a series of transporters responsible for the regulated exchange of nutrients and toxic products. These characteristics make the CNS ECs a continuous and selective physical barrier for hydrophilic substances, and a key player in the regulated trafficking of molecules into the CNS (Abbott et al. 2006) (Figure 2). Interestingly, the Blood Spinal Cord Barrier (BSCB) displays similarities to the BBB, but it also has some unique properties, among them being slightly more permeable (Bartanusz et al. 2011). Transit restrictions imposed by the BBB (and at lesser extent by BSCB) represent the most important barrier to overcome in the drug delivery to the CNS. In the context of emerging neurological diseases, targeting drugs to the CNS is under strong pushing demands, but vehicles for BBB crossing are still in their infancy, with a long run until full tailoring.

2. Cross-transportation through BBB

The BBB gradually develops in humans during the first postnatal year (Adinol 1979) and its nearly complete in rats after the second postnatal week (Stewart and Hayakawa 1987). This highly differentiated EC phenotype is

induced and maintained in the long term by interactions with the surrounding cells, mainly astrocytes and pericytes but also perivascular macrophages and even neurons (Abbott et al. 2006; Alvarez et al. 2011; Arthur et al. 1987; Janzer and Raff 1987). For instance, *in vivo*, astrocytes secrete Sonic Hedgehog (Shh), that will act on endothelial cells and promote BBB integrity (Alvarez et al. 2011). In addition to the role in long-term barrier induction and maintenance, astrocytes and other cells can release chemical factors that modulate local endothelial permeability over a time-scale of seconds to minutes. Thus, both natural stimuli for BBB leakage and pharmacological compounds acting on endogenous BBB induction pathways like Shh inhibitors (Alvarez et al. 2011) can be used to transiently increase the entrance of molecules into the CNS parenchyma. Moreover, the phenotypical characteristics of the BBB ECs includes both uptake mechanisms (e.g. GLUT-1 glucose carrier, L1 amino acid transporter, transferrin receptor) and efflux transporters (e.g. P-glycoprotein), and thus transporter/receptor-mediated transit across the BBB has also been used to deliver molecules of pharmacological interest into the CNS parenchyma (Figure 2). In this case, specific transcellular receptor-mediated transcytosis transport molecules from the luminal membrane, lining the internal surface of the vessels, to the abluminal membrane on the external CNS-lining surface. In addition, less specific adsorptive-mediated transcytosis can also be used for the delivery of molecules, but CNS ECs show a lower rate of transcytosis activity than peripheral ECs (Rubin and Staddon 1999), making this a less efficient process for the incorporation of circulating molecules.

A final consideration regarding potential limiting steps for the delivery of hydrophilic substances into the CNS across the BBB is that both intracellular and extracellular enzymes provide an additional barrier. Extracellular enzymes such as peptidases and nucleotidases are capable of metabolizing peptides and ATP respectively. Intracellular enzymes, that are involved in hepatic drug metabolism, have been found in the small microvessels from brain, the choroid plexuses, and the leptomeninges (pia plus arachnoid mater), such as monoamine oxidase and cytochrome P450, and they can inactivate many lipophilic neuroactive and toxic compounds (el-Bacha and Minn 1999).

The delivery of substances across the Blood Cerebrospinal Fluid Barrier (BCFB) may also be considered as an interesting option. This barrier shows a morphological correlate with the BBB at the level of tight junctions between the cells. These, however, are not located at the ECs capillaries that are in fact fenestrated (Figure 1 C), but on the apical surface of the epithelial cells of the choroid plexus and the arachnoid fibroblasts along the blood vessels, inhibiting paracellular diffusion of hydrophilic molecules across this barrier. When a substance reaches the cerebrospinal fluid it can diffuse through the Virchow-Robin's perivascular spaces (Bechmann et al. 2001), which are located between the basement membrane around pericytes and ECs and the basement membrane at the surface of the glia limitans of the brain vessels (Figure 1 B). These perivascular spaces are in direct contact with the subarachnoid space and thus with the cerebrospinal fluid. When small tracers are injected into the cerebrospinal fluid they follow the fluids flow through the perivascular spaces and the ventricles, and they may enter the brain parenchyma (Iliff et al. 2012). In fact, after an intracisternal injection, small hydrophilic molecules can be observed around the ventricle walls and the superficial layers of the CNS in contact with the meninges or in the whole brain parenchyma depending on the size of the molecule (Iliff et al. 2012). Larger molecules will not enter the brain parenchyma after intraventricular or intracisternal injection due to the ependymocytes and the glia limitans and its basal lamina (Bechmann et al. 2001; Iliff et al. 2012; Kim et al. 2006), being only observed in the perivascular compartment. Thus, after intravenous administration, a hydrophilic drug will not reach the cerebrospinal fluid, but if administered intracisternaly it may enter the brain parenchyma in a size-depending fashion. The engineering of appropriate vehicles for cargo drug delivery using these administration routes may be useful to envisage potential therapeutic strategies.

3. Disturbed BBB permeability

BBB disruption is a central and early characteristic of many acute and chronic CNS injuries such as stroke, trauma, inflammatory and infectious processes, Multiple Sclerosis, Alzheimer, Parkinson, epilepsy, pain, and brain tumors (Abbott et al. 2006; Rosenberg 2012). In these cases, the increase in

BBB permeability is linked to the dysfunction of the CNS (Rosenberg 2012). For instance, inflammation is a common feature of both chronic and acute CNS injuries and it is one of the main causes of the expansion of the neuropathology to adjacent CNS tissue areas. Many inflammatory mediators, like tumor necrosis factor-α (TNFα), induce BBB permeability acting directly on ECs (Deli et al. 1995) or indirectly by activating astrocytes to secrete other proinflammatory mediators like IL-1β (Didier et al. 2003), and in this way contribute to the disease severity. In the Multiple Sclerosis model termed Experimental Allergic Encephalomyelitis (EAE), the major BBB disruption occurs in white matter post-capillary venules in response to inflammatory stimuli (Tonra 2002), showing that these locations can also constitute important places for the entry of circulating molecules and cells into the brain. After a traumatic brain injury there is a rapid extravasation of blood in the central damaged areas, and intravascular coagulation and significant reduction in blood flow in the pericontusional brain areas. This is followed by two peaks of BBB opening at 4-6 hours and 2-3 days after the insult (Chodobski et al. 2011). Thus, though the extent and particular moments of BBB permeability varies in the different pathologies, it can be used as a therapeutic time-window to deliver molecules into the CNS (Rosenberg 2012).

Transient pharmacological stimulation of BBB opening for drug delivery is tempting, and it can be achieved by the injection of hypertonic solutions with Mannitol. However, the potential toxic effects, especially under pathological conditions, are notable. Though the permeability of the BBB may be spontaneously enhanced at certain time-windows post-injury, as for example after Traumatic Brain or Spinal Cord Injury (Bartanusz et al. 2011), that will allow the desired drugs entering the CNS, the pharmacological disruption of the BBB under pathological conditions may in contrast worsen the disease progression. For instance, the pharmacological disruption of the BBB enhanced the clinical severity in an EAE model (Alvarez et al. 2011), indicating that the integrity of the BBB is involved in the pathology and it also modulates the recovery. In this context, the dysfunction of the BBB and BSCB has been well documented in the etiology or progression of several CNS pathologies (Bartanusz et al. 2011), making the enhancement of BBB barrier permeability not indicated for the delivery of drugs into the damaged CNS.

Again, specific BBB crossing vehicles would be required to provide the drugs with CNS transit properties.

4. Viral and viral-based vectors for BBB crossing

Recent reports have demonstrated that some non-pathogenic, single-stranded DNA human parvoviruses, in particular the adeno-associated virus (AAV) serotypes 6 and 9, enter the CNS following intravenous (i.v.) administration without the use of any BBB-permeabilizing agents (Duque et al. 2009; Foust et al. 2009; Foust et al. 2010; Towne et al. 2008). This observation generated important expectations regarding the identification of surface protein motifs capable of inducing transport of vectors across the BBB.

Recombinant vectors for AAV-derived gene therapy (rAAVs) can infect a broad range of both dividing and post-mitotic cells, and their DNA persists in an episomal state thus enabling efficient and stable transduction (Grieger and Samulski 2005; Mandel et al. 2006). These vehicles are highly efficient in the nervous system and infect mainly neurons by intrathecal (Federici et al. 2012) or intracerebral injections (Burger et al. 2005; Mandel et al. 2006; McCown 2005). Towne and colleagues (Towne et al. 2008) observed that motor neurons could be transduced along the entire spinal cord through a single noninvasive i.v. delivery of rAAV6 in 42 days old wt and SOD1 G93A transgenic mice model of Amyotrophic Lateral Sclerosis. The transduction of astrocytes and other non-motor neuron cells, along with the finding that the motor neurons were not transduced following intramuscular injection, suggested that the mechanism of transduction was independent of retrograde transport, and that the vector was in fact able to cross the BBB (Towne et al. 2008). Moreover, rAAV9 were found to be very efficient for transducing spinal cord cells including motor neurons after i.v. delivery in both neonate and adult mice (Duque et al. 2009). Kaspar and colleagues (Foust et al. 2009) have demonstrated that delivery of rAAV9 through the systemic circulation lead to widespread transduction of the neonatal and adult mice brain, with marked differences in cell tropism in relation to the stage of development and complexity of the BBB (Foust et al. 2009; Lowenstein 2009). In accordance, Gray and colleagues (Gray et al. 2011) reported the ability of rAAV9 to

transduce neurons and glia in the brain and spinal cord of adult mice and nonhuman primates. They suggest that AAV9 enters the nervous system by an active transport mechanism across the BBB rather than by passive slipping through the tight junctions between endothelial cells, as the co-administration of mannitol prior to rAAV injection resulted in only a 50 % increase in brain delivery. They observed extensive transduction of neurons and glia throughout the mice brain and spinal cord (with neurons outnumbering astrocytes ~ 2:1 in the hippocampus and striatum and 1:1 in the cortex). However, the overall transduction efficiency was considerably lower in non-human primates, being glial cells the main cell type transduced. These rodent/non-human primate differences are important for clinical applications, and may reflect a variety of species-specific factors including differential BBB transport, capsid-interacting blood factors to promote or inhibit rAAV9 transduction, neural cell tropism within the brain, and/or intracellular trafficking and vector persistence. A summary of the AAV9 viral-based administration strategies to cross the BBB for therapeutic purposes is summarized in Figure 3. Nevertheless, the identification of the functional motifs of the surface proteins of AVV6 and AVV9 will surely contribute to the engineering of more effective vectors for the treatment of central nervous system injuries. In fact, AAV capsid DNA shuffling and subsequent directed evolution generated AVV novel clones able to cross selectively the seizure-compromised BBB after i.v. administration (Gray et al. 2010).

Obviously, in the context of biological risks associated to administration of viruses (Edelstein et al. 2007) and the inflammatory conditions linked to AVV administration and immune responses (Daya and Berns 2008), molecular carriers or non-infectious virus-inspired constructs (artificial viruses) would be preferred for drug BBB-cross delivery. Artificial viruses are nanostructured, manmade molecular oligomers that mimic viral behaviour regarding cell penetrability, targeted delivery of associated drugs and nucleic acids and other key functions relevant to encapsulation, cell surface receptor targeting, intracellular trafficking and eventual nuclear delivery, among others (Mastrobattista et al. 2006). In this regard, peptides and proteins are enough versatile to functionalize these vehicles, or the drug itself in simpler

nanoconjugates. When the building blocks of drug carries are proteins, these functions can be recruited by the incorporation, in a single polypeptide chain, of functional peptides from diverse origins that supply desired biological activities to the whole construct (Ferrer-Miralles et al. 2008; Neus Ferrer-Miralles et al. 2013; Vazquez et al. 2008; Vazquez et al. 2009). Also, principles for the rational control of self-assembling of natural and fully de novo designed polypeptides as nanostructured materials are being established (Domingo-Espin et al. 2011; Unzueta et al. 2012a; Unzueta et al. 2012b; Unzueta et al. 2013; Vazquez et al. 2010; Vazquez and Villaverde 2010), thus opening a plethora of possibilities for the design and biological production of nanostructured, protein-based artificial viruses (Neus Ferrer-Miralles et al. 2013; Rodriguez-Carmona and Villaverde 2010; Vazquez and Villaverde 2013) with good clinical grade formulation profile. The BBB-crossing abilities of AAVs prove, in any case, the potential penetrability of nanosized protein entities in the context of emerging nanomedicines of CNS.

5. BBB-crossing protein tags in artificial drug carriers

From a different angle, chemical modification of a drug can enhance its penetrability into the CNS, for example by adding domains for glycosylation (Poduslo and Curran 1992), methylation (Hansen, Jr. et al. 1992) and pegylation (Witt et al. 2001), lipophilic domains (Egleton and Davis 2005), or coating it with polysorbates (Bhaskar et al. 2010). Also, precursors can cross the BBB when the drug cannot, as is the case of L- Dopa in the treatment of Parkinson's disease (Wade and Katzman 1975). In a very different context, adequate engineering of natural proteins can offer, at different extents, tools to functionalize free drugs or nanosized carriers to reach the CNS parenchyma (Table 1). For that, receptor-mediated transcytosis can be reached by the incorporation of proteins or short peptides that act as ligands of insulin, transferrin or low density lipoprotein receptors (Table 1). For instance, monoclonal antibodies covalently bound to therapeutic proteins have been targeted to insulin and transferrin receptors (TfRs) in both *in vitro* and *in vivo* models (Fu et al. 2010b; Fu et al. 2011; Lu et al. 2011). In these experiments, recombinant proteins have two functional moieties; the therapeutic peptide fused to the carboxy terminus of the IgG heavy chain and

the complementarily determining regions of the monoclonal antibodies that are located at the N-terminus (Pardridge and Boado 2012). This delivery platform, dubbed Molecular Trojan Horse and extensively exploited by Pardridge's group (Pardridge 2006), can be adapted to any therapeutic protein as long as its production in recombinant organisms maintains its biological function. In this context, recent insights in industrial-oriented metabolic engineering (Lee et al. 2012) and the wide diversity of microbial species that are now under exploration as cell factories for therapeutic proteins (Corchero et al. 2013), offer alternatives to conventional hosts for the production of highly functional protein species. In addition, monoclonal antibodies conjugated to polymeric micelles (Yue et al. 2012), liposomes (Mamot et al. 2005; Schnyder and Huwyler 2005b; Zhang et al. 2002) and polymeric nanoparticles (Reukov et al. 2011a) can improve the performance of the chemical entities in the transport of therapeutic molecules across the BBB. Recent results suggest that low affinity binding and monovalent binding to the cellular receptors are highly effective for successful transcytosis (Niewoehner,et al., 2014; Yu et al. 2011).

In the development of photothermal therapy, gold nanoparticles conjugated to peptides carrying the motif THR target transferrin receptor (TfR) and they are delivered to the CNS (Prades et al. 2012b). Also, pegylated Fe_3O_4 nanoparticles conjugated with lactoferrin (Qiao et al. 2012b) have been proposed as MRI molecular probes for imaging diagnostic purposes. In some instances, intravenously administered nanoparticles of different chemical origin get adsorbed to apolipoproteins and the entrance to the CNS is mediated by low density lipoprotein receptors (Gessner et al. 2001; Kim et al. 2007). This is the case of human serum albumin nanoparticles (HSA) loaded with loperamide (Ulbrich et al. 2011a). Therefore, some nanoparticulate carriers have been modified to include low-density lipoproteins (LDL) or LDL receptor binding peptides (ApoB (Spencer and Verma 2007); APoE (Re et al. 2011; Wagner et al. 2012) and Apo A-I (Fioravanti et al. 2012; Kratzer et al. 2007a)) in their formulation, which results in significantly improved entrance to the brain parenchyma when compared with naked nanoparticles. In that sense, HSA nanoparticles with covalently bound ApoA-I or ApoE are able to transport drugs to the brain with similar efficiency as HSA nanoparticles

conjugated to antibodies against insulin or transferrin receptors, or HSA nanoparticles conjugated to insulin or transferrin (Zensi et al. 2009; Zensi et al. 2010). Among successful examples, peptides derived from the consensus binding sequence (Kunitz domain) of proteins transported through LDL receptors, such as aprotinin and Kunitz precursor inhibitor 1 (Demeule et al. 2008b; Gabathuler 2010b), must be stressed as very promising (Table 1). Kunitz-derived peptides (angiopeps), covalently bound to drugs, have been already used or are in ongoing clinical trials for the treatment of brain tumors. The main objective of the targeting peptides in clinics is the treatment of brain metastases from solid tumors (breast and lung cancers) as an alternative to the surgical removal of the primary brain tumor. Particularly, it has been demonstrated that angiopep conjugated to paclitaxel (ANG1005, also named GRN1005, http://clinicaltrials.gov/ct2/show/NCT01480583?term=ANG1005&rank=6), is well tolerated and shows activity in patients with advanced solid tumors previously treated with antitumor drugs (Kurzrock et al. 2012). In addition, there are three ongoing clinical trials in the same direction (http://clinicaltrials.gov). Apart from the endogenous ligands, other peptides with high affinity for brain receptors (or strong cell-penetrating peptides) have also been explored as functional materials, including pegylated-gelatin siloxane nanoparticles conjugated with HIV-1-derived Tat peptide (Tian et al. 2012), rabies virus glycoprotein conjugated to liposomes (Tao et al. 2012), variable heavy-chain domain of camel homodimeric antibodies (VHH) (Li et al., 2012) for receptor-homing peptides obtained from phage display screening (Maggie et al. 2010; Malcor et al. 2012). To gather all published information related to peptides with activity to cross the BBB, Van Dorpe and collaborators designed a peptide database to organize scattered information (Van et al. 2012) (http://brainpeps.ugent.be). The main approaches to protein-guided BBB delivery of therapeutic nanoparticles are summarized in Figure 4.

6. BBB-crossing for the treatment of CNS diseases.

Among CNS diseases, only three are currently treated with drugs that naturally cross the BBB, namely epilepsy, chronic pain and psychiatric disorders (Ghose et al. 1999). For degenerative diseases, vascular diseases,

trauma aftermaths, viral infections and congenital diseases occurring in the CNS, there is a pushing need to develop BBB-crossing strategies for drug delivery, preferentially based on non-viral carriers (Table 2). The most representative examples of how BBB-crossing is addressed in these conditions are discussed in the next sections.

6.1. Neurodegenerative disorders

Therapeutic approaches to neurodegenerative diseases are concentrating most of the efforts on the design of therapeutic compounds able to cross the BBB. For Parkinson's disease, the first drug used clinically was the dopamine precursor L-Dopa, that contrarily to dopamine itself, crosses the BBB by using a large amino acid transporter (Wade and Katzman 1975). On the other hand, in a Trojan Horse approach, Pardridge's group normalized striatal tyrosine hydroxylase levels and reversed functional signs in a Parkinson model. A tyrosine hydroxylase gene empowered by a nervous system-specific promoter was injected, carried by pegylated liposomes decorated with OX26 antibody against TfR (Zhang et al. 2003; Zhang et al. 2004a). The team was also successful entering erythropoietin (Zhou et al. 2011b) and glial derived neurotrophic factor (GDNF) (Fu et al. 2010a) by joining these therapeutic proteins to mice anti-TfR antibodies, and subsequently reaching clear neuroprotective effects.

Regarding Alzheimer, again, by means of this anti-TfR antibody as BBB transporter and by fusion to an anti-Abeta amyloid antibody, the levels of beta amyloid peptide were dramatically reduced (Zhou et al. 2011a). In this context, Genentech is developing a lower affinity variant of anti-TfR antibody (that favors release from the BBB towards the CNS) fused to an antibody against the enzyme BACE1, involved in amyloidal plaque formation. When the bifunctional molecule is applied systemically, a decrease of 47 % in plaques was observed in mouse models (Yu et al. 2011). Interestingly, the fusion of a monovalent sFab of an anti-TfR antibody to an anti-Abeta antibody mediated effective uptake transcytosis and TfR recycling, while the presence of two Fab fragments on the anti-Abeta antibody resulted in uptake followed by trafficking to lysosomes and an associated reduction in TfR

levels (Niewoehner et al, 2014). This approach exhibited enhanced *in vivo* targeting of Abeta plaques after i.v. administration. Nerve growth factor (NGF) fused to an anti-TfR antibody has also been used successfully to prevent neuronal degeneration when applied intravenously in a Huntington disease model (Kordower et al. 1994). In a similar context, a poly(mannitol-co-PEI) gene transporter modified with a rabies virus glycoprotein is able to ameliorates Alzheimer symptoms by transporting a therapeutic RNAi (Park, 2015). Alternatively, the intranasal route to the CNS (Hanson and Frey 2008), through the olfactory via and trigeminal nerve has been largely explored to introduce important factors in neurogenesis and memory such as NGF (De et al. 2005), insulin-like growth factor 1 (IGF- I) (Liu et al. 2004), fibroblast growth factor 2 (FGF-2) (Jin et al. 2003), insulin (Benedict et al. 2004), interferon beta (IFN beta (Ross et al. 2004) and the octapeptide NAP (Matsuoka et al. 2008) which is currently in Phase II clinical trials in patients with incipient Alzheimer 's disease (Gozes et al. 2009).

6.2 Brain tumors

Diverse BBB-crossing anti-tumor vectors are under development in both pre-clinical and clinical phases, empowered by a spectrum of BBB-crossing tags. Angiochem Inc. entered into Phase I clinical trials a product (ANG1005) that uses the peptide Angiopep-2, capable of driving the cargo paclitaxel by transcytosis through the BBB by using the LDL receptor LRP- 1. This conjugate showed previously intracranial tumor regression in murine models when administered i.v. (Bichat 2008). Melanotransferrin associated with doxorubicin increased the survival in mice with intracranial tumors (Gabathuler 2005; Karkan et al. 2008). Albumin is being used at University of California, San Francisco (UCSF), in a Phase I clinical trial as a carrier of paclitaxel (nab- paclitaxel) to treat brain and CNS tumors (Chien et al. 2009) (it is already in the market for breast cancer). Targeting the transmembrane protein TMEM30A, the ligand FC5 (discovered by phage display, a single domain antibody – sdAb-), drives liposomes though the BBB to release doxorubicin into CNS (Gabathuler 2010a). On the other hand, by taking a Trojan Horse strategy based on pegylated immunoliposomes targeted to TfR

(Boado et al. 2007), the delivery of shRNAs expression vectors against the epidermal growth factor receptor (EGFR) increased the survival in mice with intracranial tumors (Boado 2007; Pardridge 2004; Zhang et al. 2004b). Doxorubicin ferried by polysorbate-coated polymer nanoparticles promoted long-term glioblastoma remission in rats, probably by an unspecific BBB crossing (Steiniger et al. 2004), and a polycefin polymer variant that specifically targets human brain, which associated to antiangiogenic oligonucleotides inhibits tumor angiogenesis and improves animal survival (Ljubimova et al. 2008).

On the other hand, despite no direct CNS targeting, it has been possible to increase the intracranial levels of anticancer 3'5'-dioctanoyl-5-fluoro-2'deoxyuridine (DO-FUdR), by incorporating it into a solid lipid nanoparticle (Wang et al. 2002). Furthermore, when administered systemically, nude phosphorothioate oligonucleotides against protein kinase C alpha, also reduced intracranial glioblastoma tumor size and doubled mice survival time (Yazaki et al. 1996). On the basis of these results, a phase II clinical trial has been completed (http://www.clinicaltrials.gov/ct2/results?term=pkc-alpha). In a more recent example, an intravenously injected cell penetrating peptide (LNP) decorating a polylysine-PEG gene vector extended the median survival time of glioma-bearing mice (Yao et al. 2014).

6.3 Pain

Anti-nociception is usually achieved by methylation (Hansen, Jr. et al. 1992) or glycosylation (Polt et al. 1994) of active molecules to stimulate their penetrability into the CNS. On the other hand, coupling human serum albumin to an anti-TfR permits the transport of loperamide into the CNS for anti-nociception effects (Ulbrich et al. 2009). The same drug is delivered into the CNS by injecting i.v. a poly(lactic-co-glycolic) acid (PLGA) nanoparticle, derivatized with the peptide H_2N-Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O-β-D-Glucose)-$CONH_2$ (g7) (Tosi et al. 2007). The analgesic dalargine joined to a cationic cell-penetrating peptide (Syn–B) increases brain uptake in two orders of magnitude. This peptide crosses the BBB using a nonspecific route, that is, without association with a receptor (Rousselle et al. 2003). Other

polyarginine-based peptides as CNS transporters are in preclinical phases (Gabathuler 2010a).

6.4. Ischemia

Sequelae of cerebral ischemia can be lessened by CNS deliver of brain-derived neurotrophic factor (BDNF) (Wu and Pardridge 1999; Zhang and Pardridge 2001), fibroblast growth factor (FGF-2) (Song et al. 2002), inhibitor of caspase-3 (Yemisci et al, 2014), vasoactive intestinal peptide (VIP) (Bickel et al. 1993; Wu and Pardridge 1996) and erythropoietin (EPO) (Fu et al. 2011) linked to an anti-TfR antibody. The nerve growth factor (NGF) gene has been introduced into the CNS while inside lipoplexes decorated with the TfR natural ligand, transferrin (da Cruz et al. 2005). The cell penetrating Tat peptide has also proven to carry efficiently N-methyl D-aspartate receptor subtype 2B (NR2B) domain (Aarts et al. 2002), B-cell lymphoma-extra large protein (Bcl-X_L) (Kilic et al. 2002), glial cell-derived neurotrophic factor (GDNF) (Kilic et al. 2003) and c-Jun domain (Borsello et al. 2003), to protect neurons in brain infarct models. On the other side, sniffing insulin-like growth factor (IGF-1) (Liu et al. 2004) and EPO (Yu et al. 2005) protects brain against stroke in animal models (Hanson and Frey 2008). Modular protein/DNA nanoparticles have been shown to induce biologically relevant transgenic protein levels and therapeutic effects after acute excytotoxic injuries when injected intracerebrally (Negro-Demontel,et al., 2014; Peluffo et al. 2003; Peluffo et al. 2006; Peluffo et al. 2011). The addition of CNS targeting domains to these particles may enable intravenous delivery retaining its neuroprotective potential.

6.5 Infectious diseases

CNS infectious diseases have also been treated *in vivo* using different approaches. By administering i.v. siRNA into Japanese encephalitis virus-infected mice, Manjunath and cols. afforded specific viral gene silencing and protection. The siRNA carrier was a two-domain peptide formed by nine arginines (R9) and a peptide derived from rabies virus glycoprotein (RVG) (Kumar et al. 2007). On the other hand, the brain levels of different anti HIV drugs have been increased several folds through association with liposomes

485 (foscarnet, (Dusserre et al. 1995)), micelles (zidovudine, lamivudine,
486 nelfinavir, (Spitzenberger et al. 2007)) and the Tat protein (ritonavir, (Rao et
487 al. 2009)). Furthermore, second stage African trypanosomiasis was treated
488 intravenously in a mouse model by conjugating the active water-soluble drug
489 to liposomes using polysorbate 80 as surfactant (Olbrich et al. 2002).
490
491 *6.6. Other conditions*
492 Other diseases in which the BBB crossing has been successfully
493 achieved are Hurler's Syndrome (mucopolysacharidosis), using the mouse
494 anti-TfR antibody associated to a liposome with beta-glucuronidase gene
495 (Zhang et al. 2008) or fusioned to the alpha-L-iduronidase enzyme (Boado et
496 al. 2008). A cell-penetrating Tat peptide improves the beta-glucuronidase
497 biodistribution when organized as a single chain fusion protein (Xia et al.
498 2001). Narcolepsy has also been treated with good results with nasal
499 hypocretin I (Hanson and Lobner 2004).
500
501
502 **7. Administration routes**
503 The intravenous administration of functionalized nanoparticles is the
504 most used therapeutic route. However, in some cases, patient compliance is
505 not easy to achieve, and alternative administration routes need to be
506 explored. In fact, there are standardized methods for drug delivery by osmotic
507 disruption (Kroll and Neuwelt 1998; Yang et al. 2011), by local delivery placing
508 polymer wafers after tumor excision (Balossier et al. 2010), by convection-
509 enhanced delivery (White et al. 2012a; White et al. 2012b) or by intranasal
510 administration (Grassin-Delyle et al. 2012; Tsai 2012; Wolf et al. 2012; Zhu et
511 al. 2012) (Figure 1 A). Some of these treatments are still highly invasive and
512 are only addressed to high grade glioma patients. In the milder intranasal
513 delivery, the drug is being accumulated in the olfactive bulb and then diffusing
514 inside the brain. This approach has been proven to be quite effective in the
515 treatment of various disease models, acting through the olfactory pathway
516 and trigeminal nerve (Born et al. 2002; Hanson and Frey 2008). Regarding
517 gene therapy, only 1.9 % of current clinical trials are performed on the CNS,
518 and almost all of them are applied by intracranial injection or performed ex

vivo (Ginn et al. 2013), pointing to the importance of the delivery of BBB-crossing gene therapy vectors.

8. Conclusions and future prospects

Numerous examples of basic research and ongoing clinical trials illustrate how proteins can be engineered to overcome the complexity of both BBB and BSCB in drug delivery contexts. In this regard, a few CNS diseases are already treated with protein-based targeted drugs, and much more are expected to be released for use in the next future. Hopefully, and based on current insights on the engineering of protein self-assembling, functional proteins would be desirably adapted as building blocks of nanosized entities, acting at the same time as BBB crossers, targeting agents and drug carriers. Although the fully de novo design of such protein-based artificial viruses is in its infancy, the accumulation of data about the physiology of the CNS and of relevant cell receptors, the widening spectrum of drugs potentially useful in CNS therapies and the exploration of alternative routes for administration on the bases of result from the use of natural viruses envisage the generation of these sophisticated vehicles as a forthcoming routine strategy.

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Legends:

Figure 1. Anatomical basis of the BBB. Boundaries of the CNS tissue contacting the blood vessels, meninges and the cerebrospinal fluid are depicted (A), and also alternative routes for administration of substances to the CNS to bypass the BBB. The intimate relationship between ECs, continuous basement membrane, astrocytes, pericytes and perivascular macrophages contributing to various degrees to the BBB formation and maintenance can be observed (B). Moreover, ependimocytes of the choroid plexus produce the cerebrospinal fluid and conform, in addition, the Blood Cerebrospinal Fluid Barrier (BCFB) (C).

Figure 2. Main barriers and transport mechanisms of the BBB. Physical barriers as endothelial cell membranes or intercellular tight junctions are the principal obstacles to overcome for polar macromolecules to enter the CNS (left). Moreover, intracellular and extracellular enzymes, basal membrane and astrocyte endfeet can also constitute additional barriers. Endogenous protein mediated selective transport mechanisms for small polar substances and macromolecules are the responsible for the communication of the CNS with the blood flow (right). These can be exploited for targeted delivery of different types of nanocomplexes.

Figure 3. AAV9 administration routes and transduction efficiencies. Different results have been obtained when AAV9 where administered by i.v. or intra-thecal delivery, but also in postnatal or adult animals, and importantly in mice or in non-human primates. While i.v. delivery efficiently transduce neurons and astrocytes in postnatal and adult mice, very low efficiency and mainly astrocyte transduction was observed in non-human primates. Moreover, intrathecal delivery into the Cisterna Magna resulted in the widest transduction in non-human primates.

Figure 4. Receptor-mediated approaches used in Nanomedicine to cross the BBB. Different types of proteins (including antibodies) showing specific binding to BBB transporters and cell surface receptors that are relevant to transcytosis are used to functionalize nanoparticles (NPs). Cell-penetrating

peptides carrying therapeutic proteins are also depicted. More details and specific examples are given in Table 1.

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591 Table 1. Main transversal approaches to address BBB-crossing in Nanomedicine, illustrated by representative examples.

592

Method	**Target**	**Ligand and references**	**Application and reference**	**NP size**
Therapeutic proteins conjugated to mAbs raised against insulin and transferrin receptors	Transferrin receptor	Carboxy terminus of the IgG heavy chain(mAb) against the mouse transferrin receptor	Erythropoietin fused to the mAb to treat Stroke (Fu et al. 2011)	ND
	Insulin receptor	Monoclonal antibodies conjugated to polymeric micelles, liposomes (Mamot et al. 2005a; Schnyder and Huwyler 2005a; Ulbrich et al. 2011b) and polymeric nanoparticles (Reukov et al. 2011b) against insulin receptor	Insulin or an anti-insulin receptor mAbs were covalently coupled to the Human serum albumin NP (Zensi et al. 2010a)	157±11 nm
Adsorption of apolipoproteins on chemical NPs to interact with LDLR	LDLR	Apolipoproteins	Adsorption of apolipoprotein B-100 (ApoB-100) onto PEG-PHDCA NPs (Kim et al. 2007a)	135 ±41 nm

Conjugation or covalent binding of endogenous ligands (proteins or peptides) to nanocarriers	Transferrin receptor	THR derived peptide	Gold nanoparticles conjugated to THR peptide target transferrin receptor and can deliver gold NPs to the CNS (Prades et al. 2012a)	519±10 nm
	Transferrin receptor	Lactoferrin	Pegylated Fe_3O_4 NPS conjugated with lactoferrin used for imaging diagnostic purposes (Qiao et al. 2012a)	48.9 nm
	LDLR	Peptides derived from ApoE[20,29], ApoB[23] and ApoA-I (Kratzer et al. 2007b; Lu et al. 2011a)	LDLR binding-domain of ApoB was cloned into lentivirus vector (Spencer and Verma 2007a)	ND
	LDLR	Peptides originated from Kunitz protein (angiopeps)	Covalently bound to drugs used for the treatment of brain tumors (Demeule et al. 2008a)	ND

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594 mAbs: monoclonal antibodies
595 LDLR: low density liproprotein receptor
596 Apo: apolipoprotein
597 NP: nanoparticle
598 ND: not determined
599 THR: tri-peptide motif (thre-his-arg)
600

601 Table 2: Disease-focused main approaches to BBB drug transdelivery.

Disease	Drug	Target	Ligand and strategy	References
Neurodegenerative disorders				
Parkinson	L-Dopa	Large amino acid transporter	L-dopa	(Wade and Katzman 1975)
	Tyrosine hydroxylase gene	TfR	Pegylated liposome decorated with OX26 ab agains TfR.	(Zhang et al. 2003; Zhang et al. 20
	Erythropoietin	TfR	Fusion protein joined to TfR ab.	(Zhou et al. 2011b)
	GDNF	TfR	Fusion protein joined to TfR ab.	(Fu et al. 2010b)
Alzheimer	Ab against beta-amyloid	TfR	Fusion protein joined to TfR ab.	(Zhou et al. 2011a)
	Ab against BACE1 enzyme	TfR	Fusion protein joined to low affinity TfR ab.	(Yu et al. 2011)
Huntinton disease	NGF	TfR	Fusion protein joined to TfR ab.	(Kordower et al. 1994)
Brain tumors				
	Antiangiogenic oligonucleotides	ND	Polycefin polymer	(Ljubimova et al. 2008)
	DO-FUdR	ND	Drug incorporated in solid lipid nanoparticles	(Wang et al. 2002)
Intracranial tumor	Paclitaxel	LRP-1 (LDL receptor)	Drug conjugated to Angiopep-2 peptide.	(Bichat 2008)
	Paclitaxel	Melanotransferrin	Drug associated with Melanotransferrin	(Karkan et al. 2008)

		receptor		
	Paclitaxel	ND	Drug conjugated to Albumin	(Chien et al. 2009)
	Doxorubicin	TMEM30A transmembrane protein	Liposomes decorated with FC5 ligand	(Gabathuler 2010a)
	shRNAs against EGFR	Insuline Receptor / Transferrine receptor	Pegylated immunolyposomes associated to TfR ab and Insulin receptor Ab.	(Boado 2007; Pardridge 2004)
	Doxorubicin	LDL receptor via ApoB/E enrichment	Drug bound to polysorbate-coated polymer	(Steiniger et al. 2004)
	Oligonucleotides against protein kinase C alpha	ND	Nude oligonucleotide administration	(Yazaki et al. 1996)
Anti-nociception				
	Loperamide	TfR	Human serum albumin coupled to TfR ab.	(Ulbrich et al. 2009)
	Loperamide	Possible adsorption-mediated endocytosis	PLGA nanoparticle derivatized with a glicosylated heptapeptide	(Tosi et al. 2007)
	Dalargine	ND	Drug joined to cell penetrating peptides	(Rousselle et al. 2003)
	Dalargine	TMEM30A transmembrane protein	Drug joined to a FC5-Fc fusion antibody	(Farrington et al. 2014)
Cerebral isquemia				

	BDNF	TfR	Protein linked to TfR ab.	(Wu and Pardridge 1999)
	FGF-2	TfR	Protein linked to TfR ab.	(Song et al. 2002)
	VIP	TfR	Protein linked to TfR ab.	(Bickel et al. 1993)
	Erythropoietin	TfR	Protein linked to TfR ab.	(Fu et al. 2011)
	NGF gene	TfR	Lipoplexes decorated with transferrin	(da Cruz et al. 2005)
	NR2B	ND	Protein fused to cell penetrating peptide	(Aarts et al. 2002)
	Bcl-Xl	ND	Protein fused to cell penetrating peptide	(Kilic et al. 2002)
	GDNF	ND	Protein fused to cell penetrating peptide	(Kilic et al. 2003)
	JNKI	ND	Protein fused to cell penetrating peptide	(Borsello et al. 2003)
Infectious diseases				
	siRNA	ND	9R-RVG fusion protein	(Kumar et al. 2007)
	Anti-VIH drugs	ND	Drug associated to liposomes	(Dusserre et al. 1995)
	Anti-VIH drugs	ND	Drug associated to micelles	(Spitzenberger et al. 2007)
	Anti-VIH drugs	ND	Drug associated to cell penetrating peptide	(Rao et al. 2009)
	Diminazenediaceturate	LDL receptor via Apo E enrichement	Lipid-drug conjugate	(Gessner et al. 2001)
Mucopolysacharidosis				
	Beta-glucuronidase gene	TfR	Liposomes associated to TfR Ab.	(Zhang et al. 2008)
	Alpha-L-iduronidase enzyme	TfR	Protein linked to TfR ab.	(Boado et al. 2008)
	Beta-glucuronidase	ND	Protein fused to cell penetrating peptide	(Xia et al. 2001)

602 ND: not determined
603 PLGA: Poly(lactic-co-glycolic) acid

TfR: Transferrin receptor

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