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4BBB-targeting, protein-based nanomedicines for drug 5and nucleic acid delivery to the CNS

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23Abstract

24The increasing incidence of diseases affecting the central nervous system
25(CNS) demands the urgent development of efficient drugs. While many of
26these medicines are already available, the Blood Brain Barrier and to a lesser
27extent, the Blood Spinal Cord Barrier pose physical and biological limitations
28to their diffusion to reach target tissues. Therefore, efforts are needed not only
29to address drug development but specially to design suitable vehicles for
30delivery into the CNS through systemic administration. In the context of the
31functional and structural versatility of proteins, recent advances in their
32biological fabrication and a better comprehension of the physiology of the
33CNS offer a plethora of opportunities for the construction and tailoring of plain
34nanoconjugates and of more complex nanosized vehicles able to cross these
35barriers. We revise here how the engineering of functional proteins offer drug
36delivery tools for specific CNS diseases and more transversally, how proteins
37can be engineered into smart nanoparticles or ‘artificial viruses’ to afford
38therapeutic requirements through alternative administration routes.

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40

41Keywords: Nanoparticles; BBB; Protein engineering; Recombinant proteins;
42Artificial viruses; Drug delivery; Gene therapy

43

441. Introduction

45 The maintenance of the central nervous system (CNS) homeostasis is
46 essential for its normal function. The limits of the CNS tissue are established
47 by the astrocytic glia limitans facing the meninges and the blood vessels, and
48 by the ependimocytes of the choroid plexus where the cerebrospinal fluid is
49 produced (Figure 1 A). Astrocyte end-feet wrap the meningeal fibroblasts and
50 the endothelial cells (ECs) of the capillaries, leaving between them the
51 basement membrane. Brain capillaries display a large surface area ($\sim 20 \text{ m}^2$
52 per 1.3 kg brain), and thus possess a predominant role in regulating the brain
53 microenvironment. The blood-brain-barrier (BBB) limits the entry of blood-
54 derived molecules and circulating leukocytes, protecting the CNS from
55 fluctuations in plasma compositions or circulating agents such as
56 neurotransmitters and xenobiotics. It is composed of specialized ECs held
57 together by multiprotein complexes known as tight junctions, astrocytes,
58 pericytes and basement membrane (Abbott et al. 2006; Reese and Karnovsky
59 1967) (Figure 1 B). CNS ECs display more efficient cell-to-cell tight junctions
60 than other ECs (Wolburg and Lippoldt 2002), rest on a continuous basement
61 membrane and express a series of transporters responsible for the regulated
62 exchange of nutrients and toxic products. These characteristics make the
63 CNS ECs a continuous and selective physical barrier for hydrophilic
64 substances, and a key player in the regulated trafficking of molecules into the
65 CNS (Abbott et al. 2006) (Figure 2). Interestingly, the Blood Spinal Cord
66 Barrier (BSCB) displays similarities to the BBB, but it also has some unique
67 properties, among them being slightly more permeable (Bartanusz et al.
68 2011). Transit restrictions imposed by the BBB (and at lesser extent by BSCB)
69 represent the most important barrier to overcome in the drug delivery to the
70 CNS. In the context of emerging neurological diseases, targeting drugs to the
71 CNS is under strong pushing demands, but vehicles for BBB crossing are still
72 in their infancy, with a long run until full tailoring.

73

742. Cross-transportation through BBB

75 The BBB gradually develops in humans during the first postnatal year
76 (Adinolfi 1979) and its nearly complete in rats after the second postnatal week
77 (Stewart and Hayakawa 1987). This highly differentiated EC phenotype is
78 induced and maintained in the long term by interactions with the surrounding

79cells, mainly astrocytes and pericytes but also perivascular macrophages and
80even neurons (Abbott et al. 2006; Alvarez et al. 2011; Arthur et al. 1987;
81Janzer and Raff 1987). For instance, *in vivo*, astrocytes secrete Sonic
82Hedgehog (Shh), that will act on endothelial cells and promote BBB integrity
83(Alvarez et al. 2011). In addition to the role in long-term barrier induction and
84maintenance, astrocytes and other cells can release chemical factors that
85modulate local endothelial permeability over a time-scale of seconds to
86minutes. Thus, both natural stimuli for BBB leakage and pharmacological
87compounds acting on endogenous BBB induction pathways like Shh inhibitors
88(Alvarez et al. 2011) can be used to transiently increase the entrance of
89molecules into the CNS parenchyma. Moreover, the phenotypical
90characteristics of the BBB ECs includes both uptake mechanisms (e.g. GLUT-
91 glucose carrier, L1 amino acid transporter, transferrin receptor) and efflux
92transporters (e.g. P-glycoprotein), and thus transporter/receptor-mediated
93transit across the BBB has also been used to deliver molecules of
94pharmacological interest into the CNS parenchyma (Figure 2). In this case,
95specific transcellular receptor-mediated transcytosis transport molecules from
96the luminal membrane, lining the internal surface of the vessels, to the
97abluminal membrane on the external CNS-lining surface. In addition, less
98specific adsorptive-mediated transcytosis can also be used for the delivery of
99molecules, but CNS ECs show a lower rate of transcytosis activity than
100peripheral ECs (Rubin and Staddon 1999), making this a less efficient
101process for the incorporation of circulating molecules.

102 A final consideration regarding potential limiting steps for the delivery of
103hydrophilic substances into the CNS across the BBB is that both intracellular
104and extracellular enzymes provide an additional barrier. Extracellular enzymes
105such as peptidases and nucleotidases are capable of metabolizing peptides
106and ATP respectively. Intracellular enzymes, that are involved in hepatic drug
107metabolism, have been found in the small microvessels from brain, the
108choroid plexuses, and the leptomeninges (pia plus arachnoid mater), such as
109monoamine oxidase and cytochrome P450, and they can inactivate many
110lipophilic neuroactive and toxic compounds (el-Bacha and Minn 1999).

111 The delivery of substances across the Blood Cerebrospinal Fluid
112Barrier (BCFB) may also be considered as an interesting option. This barrier

113 shows a morphological correlate with the BBB at the level of tight junctions
114 between the cells. These, however, are not located at the ECs capillaries that
115 are in fact fenestrated (Figure 1 C), but on the apical surface of the epithelial
116 cells of the choroid plexus and the arachnoid fibroblasts along the blood
117 vessels, inhibiting paracellular diffusion of hydrophilic molecules across this
118 barrier. When a substance reaches the cerebrospinal fluid it can diffuse
119 through the Virchow-Robin's perivascular spaces (Bechmann et al. 2001),
120 which are located between the basement membrane around pericytes and
121 ECs and the basement membrane at the surface of the glia limitans of the
122 brain vessels (Figure 1 B). These perivascular spaces are in direct contact
123 with the subarachnoid space and thus with the cerebrospinal fluid. When
124 small tracers are injected into the cerebrospinal fluid they follow the fluids flow
125 through the perivascular spaces and the ventricles, and they may enter the
126 brain parenchyma (Ilf et al. 2012). In fact, after an intracisternal injection,
127 small hydrophilic molecules can be observed around the ventricle walls and
128 the superficial layers of the CNS in contact with the meninges or in the whole
129 brain parenchyma depending on the size of the molecule (Ilf et al. 2012).
130 Larger molecules will not enter the brain parenchyma after intraventricular or
131 intracisternal injection due to the ependymocytes and the glia limitans and its
132 basal lamina (Bechmann et al. 2001; Ilf et al. 2012; Kim et al. 2006) , being
133 only observed in the perivascular compartment. Thus, after intravenous
134 administration, a hydrophilic drug will not reach the cerebrospinal fluid, but if
135 administered intracisternally it may enter the brain parenchyma in a size-
136 depending fashion. The engineering of appropriate vehicles for cargo drug
137 delivery using these administration routes may be useful to envisage potential
138 therapeutic strategies.

139

1403. Disturbed BBB permeability

141 BBB disruption is a central and early characteristic of many acute and
142 chronic CNS injuries such as stroke, trauma, inflammatory and infectious
143 processes, Multiple Sclerosis, Alzheimer, Parkinson, epilepsy, pain, and brain
144 tumors (Abbott et al. 2006; Rosenberg 2012). In these cases, the increase in
145 BBB permeability is linked to the dysfunction of the CNS (Rosenberg 2012).
146 For instance, inflammation is a common feature of both chronic and acute
147 CNS injuries and it is one of the main causes of the expansion of the

148neuropathology to adjacent CNS tissue areas. Many inflammatory mediators,
149like tumor necrosis factor- α (TNF α), induce BBB permeability acting directly
150on ECs (Deli et al. 1995) or indirectly by activating astrocytes to secrete other
151proinflammatory mediators like IL-1 β (Didier et al. 2003), and in this way
152contribute to the disease severity. In the Multiple Sclerosis model termed
153Experimental Allergic Encephalomyelitis (EAE), the major BBB disruption
154occurs in white matter post-capillary venules in response to inflammatory
155stimuli (Tonra 2002), showing that these locations can also constitute
156important places for the entry of circulating molecules and cells into the brain.
157After a traumatic brain injury there is a rapid extravasation of blood in the
158central damaged areas, and intravascular coagulation and significant
159reduction in blood flow in the pericontusional brain areas. This is followed by
160two peaks of BBB opening at 4-6 hours and 2-3 days after the insult
161(Chodobski et al. 2011). Thus, though the extent and particular moments of
162BBB permeability varies in the different pathologies, it can be used as a
163therapeutic time-window to deliver molecules into the CNS (Rosenberg 2012).
164 Transient pharmacological stimulation of BBB opening for drug delivery
165is tempting, and it can be achieved by the injection of hypertonic solutions
166with Mannitol. However, the potential toxic effects, especially under
167pathological conditions, are notable. Though the permeability of the BBB may
168be spontaneously enhanced at certain time-windows post-injury, as for
169example after Traumatic Brain or Spinal Cord Injury (Bartanusz et al. 2011),
170that will allow the desired drugs entering the CNS, the pharmacological
171disruption of the BBB under pathological conditions may in contrast worsen
172the disease progression. For instance, the pharmacological disruption of the
173BBB enhanced the clinical severity in an EAE model (Alvarez et al. 2011),
174indicating that the integrity of the BBB is involved in the pathology and it also
175modulates the recovery. In this context, the dysfunction of the BBB and BSCB
176has been well documented in the etiology or progression of several CNS
177pathologies (Bartanusz et al. 2011), making the enhancement of BBB barrier
178permeability not indicated for the delivery of drugs into the damaged CNS.
179Again, specific BBB crossing vehicles would be required to provide the drugs
180with CNS transit properties.

181

1824. **Viral and viral-based vectors for BBB crossing**

183 Recent reports have demonstrated that some non-pathogenic, single-
184stranded DNA human parvoviruses, in particular the adeno-associated virus
185(AAV) serotypes 6 and 9, enter the CNS following intravenous (i.v.)
186administration without the use of any BBB-permeabilizing agents (Duque et al.
1872009; Foust et al. 2009; Foust et al. 2010; Towne et al. 2008). This
188observation generated important expectations regarding the identification of
189surface protein motifs capable of inducing transport of vectors across the
190BBB.

191 Recombinant vectors for AAV-derived gene therapy (rAAVs) can infect
192a broad range of both dividing and post-mitotic cells, and their DNA persists in
193an episomal state thus enabling efficient and stable transduction (Grieger and
194Samulski 2005; Mandel et al. 2006). These vehicles are highly efficient in the
195nervous system and infect mainly neurons by intrathecal (Federici et al. 2012)
196or intracerebral injections (Burger et al. 2005; Mandel et al. 2006; McCown
1972005). Towne and colleagues (Towne et al. 2008) observed that motor
198neurons could be transduced along the entire spinal cord through a single
199noninvasive i.v. delivery of rAAV6 in 42 days old wt and SOD1 G93A
200transgenic mice model of Amyotrophic Lateral Sclerosis. The transduction of
201astrocytes and other non-motor neuron cells, along with the finding that the
202motor neurons were not transduced following intramuscular injection,
203suggested that the mechanism of transduction was independent of retrograde
204transport, and that the vector was in fact able to cross the BBB (Towne et al.
2052008). Moreover, rAAV9 were found to be very efficient for transducing spinal
206cord cells including motor neurons after i.v. delivery in both neonate and adult
207mice (Duque et al. 2009). Kaspar and colleagues (Foust et al. 2009) have
208demonstrated that delivery of rAAV9 through the systemic circulation lead to
209widespread transduction of the neonatal and adult mice brain, with marked
210differences in cell tropism in relation to the stage of development and
211complexity of the BBB (Foust et al. 2009; Lowenstein 2009). In accordance,
212Gray and colleagues (Gray et al. 2011) reported the ability of rAAV9 to
213transduce neurons and glia in the brain and spinal cord of adult mice and
214nonhuman primates. They suggest that AAV9 enters the nervous system by
215an active transport mechanism across the BBB rather than by passive slipping

216through the tight junctions between endothelial cells, as the co-administration
217of mannitol prior to rAAV injection resulted in only a 50 % increase in brain
218delivery. They observed extensive transduction of neurons and glia throughout
219the mice brain and spinal cord (with neurons outnumbering astrocytes ~ 2:1 in
220the hippocampus and striatum and 1:1 in the cortex). However, the overall
221transduction efficiency was considerably lower in non-human primates, being
222glial cells the main cell type transduced. These rodent/non-human primate
223differences are important for clinical applications, and may reflect a variety of
224species-specific factors including differential BBB transport, capsid-interacting
225blood factors to promote or inhibit rAAV9 transduction, neural cell tropism
226within the brain, and/or intracellular trafficking and vector persistence. A
227summary of the AAV9 viral-based administration strategies to cross the BBB
228for therapeutic purposes is summarized in Figure 3. Nevertheless, the
229identification of the functional motifs of the surface proteins of AVV6 and AVV9
230will surely contribute to the engineering of more effective vectors for the
231treatment of central nervous system injuries. In fact, AAV capsid DNA shuffling
232and subsequent directed evolution generated AVV novel clones able to cross
233selectively the seizure-compromised BBB after i.v. administration (Gray et al.
2342010).

235

236 Obviously, in the context of biological risks associated to administration
237of viruses (Edelstein et al. 2007) and the inflammatory conditions linked to
238AVV administration and immune responses (Daya and Berns 2008),
239molecular carriers or non-infectious virus-inspired constructs (artificial viruses)
240would be preferred for drug BBB-cross delivery. Artificial viruses are
241nanostructured, manmade molecular oligomers that mimic viral behaviour
242regarding cell penetrability, targeted delivery of associated drugs and nucleic
243acids and other key functions relevant to encapsulation, cell surface receptor
244targeting, intracellular trafficking and eventual nuclear delivery, among others
245(Mastrobattista et al. 2006). In this regard, peptides and proteins are enough
246versatile to functionalize these vehicles, or the drug itself in simpler
247nanoconjugates. When the building blocks of drug carriers are proteins, these
248functions can be recruited by the incorporation, in a single polypeptide chain,
249of functional peptides from diverse origins that supply desired biological

250activities to the whole construct (Ferrer-Miralles et al. 2008; Neus Ferrer-
251Miralles et al. 2013; Vazquez et al. 2008; Vazquez et al. 2009). Also, principles
252for the rational control of self-assembling of natural and fully de novo
253designed polypeptides as nanostructured materials are being established
254(Domingo-Espin et al. 2011; Unzueta et al. 2012a; Unzueta et al. 2012b;
255Unzueta et al. 2013; Vazquez et al. 2010; Vazquez and Villaverde 2010) , thus
256opening a plethora of possibilities for the design and biological production of
257nanostructured, protein-based artificial viruses (Neus Ferrer-Miralles et al.
2582013; Rodriguez-Carmona and Villaverde 2010; Vazquez and Villaverde
2592013) with good clinical grade formulation profile. The BBB-crossing abilities
260of AAVs prove, in any case, the potential penetrability of nanosized protein
261entities in the context of emerging nanomedicines of CNS.

262

2635. **BBB-crossing protein tags in artificial drug carriers**

264 From a different angle, chemical modification of a drug can enhance its
265penetrability into the CNS, for example by adding domains for glycosylation
266(Poduslo and Curran 1992), methylation (Hansen, Jr. et al. 1992) and
267pegylation (Witt et al. 2001), lipophilic domains (Egleton and Davis 2005), or
268coating it with polysorbates (Bhaskar et al. 2010). Also, precursors can cross
269the BBB when the drug cannot, as is the case of L- Dopa in the treatment of
270Parkinson's disease (Wade and Katzman 1975). In a very different context,
271adequate engineering of natural proteins can offer, at different extents, tools to
272functionalize free drugs or nanosized carriers to reach the CNS parenchyma
273(Table 1). For that, receptor-mediated transcytosis can be reached by the
274incorporation of proteins or short peptides that act as ligands of insulin,
275transferrin or low density lipoprotein receptors (Table 1). For instance,
276monoclonal antibodies covalently bound to therapeutic proteins have been
277targeted to insulin and transferrin receptors (TfRs) in both *in vitro* and *in vivo*
278models (Fu et al. 2010b; Fu et al. 2011; Lu et al. 2011) . In these experiments,
279recombinant proteins have two functional moieties; the therapeutic peptide
280fused to the carboxy terminus of the IgG heavy chain and the complementarily
281determining regions of the monoclonal antibodies that are located at the N-
282terminus (Pardridge and Boado 2012). This delivery platform, dubbed
283Molecular Trojan Horse and extensively exploited by Pardridge's group

284(Pardridge 2006), can be adapted to any therapeutic protein as long as its
285production in recombinant organisms maintains its biological function. In this
286context, recent insights in industrial-oriented metabolic engineering (Lee et al.
2872012) and the wide diversity of microbial species that are now under
288exploration as cell factories for therapeutic proteins (Corchero et al. 2013),
289offer alternatives to conventional hosts for the production of highly functional
290protein species. In addition, monoclonal antibodies conjugated to polymeric
291micelles (Yue et al. 2012), liposomes (Mamot et al. 2005; Schnyder and
292Huwyler 2005b; Zhang et al. 2002) and polymeric nanoparticles (Reukov et al.
2932011a) can improve the performance of the chemical entities in the transport
294of therapeutic molecules across the BBB. Recent results suggest that low
295affinity binding and monovalent binding to the cellular receptors are highly
296effective for successful transcytosis (Niewoehner,et al., 2014; Yu et al. 2011).

297 In the development of photothermal therapy, gold nanoparticles
298conjugated to peptides carrying the motif THR target transferrin receptor (TfR)
299and they are delivered to the CNS (Prades et al. 2012b). Also, pegylated
300Fe₃O₄ nanoparticles conjugated with lactoferrin (Qiao et al. 2012b) have been
301proposed as MRI molecular probes for imaging diagnostic purposes. In some
302instances, intravenously administered nanoparticles of different chemical
303origin get adsorbed to apolipoproteins and the entrance to the CNS is
304mediated by low density lipoprotein receptors (Gessner et al. 2001; Kim et al.
3052007). This is the case of human serum albumin nanoparticles (HSA) loaded
306with loperamide (Ulbrich et al. 2011a). Therefore, some nanoparticulate
307carriers have been modified to include low-density lipoproteins (LDL) or LDL
308receptor binding peptides (ApoB (Spencer and Verma 2007); APoE (Re et al.
3092011; Wagner et al. 2012) and Apo A-I (Fioravanti et al. 2012; Kratzer et al.
3102007a)) in their formulation, which results in significantly improved entrance to
311the brain parenchyma when compared with naked nanoparticles. In that
312sense, HSA nanoparticles with covalently bound ApoA-I or ApoE are able to
313transport drugs to the brain with similar efficiency as HSA nanoparticles
314conjugated to antibodies against insulin or transferrin receptors, or HSA
315nanoparticles conjugated to insulin or transferrin (Zensi et al. 2009; Zensi et
316al. 2010). Among successful examples, peptides derived from the consensus
317binding sequence (Kunitz domain) of proteins transported through LDL

318receptors, such as aprotinin and Kunitz precursor inhibitor 1 (Demeule et al.
3192008b; Gabathuler 2010b), must be stressed as very promising (Table 1).
320Kunitz-derived peptides (angiopeps), covalently bound to drugs, have been
321already used or are in ongoing clinical trials for the treatment of brain tumors.
322The main objective of the targeting peptides in clinics is the treatment of brain
323metastases from solid tumors (breast and lung cancers) as an alternative to
324the surgical removal of the primary brain tumor. Particularly, it has been
325demonstrated that angiopep conjugated to paclitaxel (ANG1005, also named
326GRN1005, [http://clinicaltrials.gov/ct2/show/NCT01480583?](http://clinicaltrials.gov/ct2/show/NCT01480583?term=ANG1005&rank=6)
327[term=ANG1005&rank=6](http://clinicaltrials.gov/ct2/show/NCT01480583?term=ANG1005&rank=6)), is well tolerated and shows activity in patients with
328advanced solid tumors previously treated with antitumor drugs (Kurzrock et al.
3292012). In addition, there are three ongoing clinical trials in the same direction
330(<http://clinicaltrials.gov>). Apart from the endogenous ligands, other peptides
331with high affinity for brain receptors (or strong cell-penetrating peptides) have
332also been explored as functional materials, including pegylated-gelatin
333siloxane nanoparticles conjugated with HIV-1-derived Tat peptide (Tian et al.
3342012), rabies virus glycoprotein conjugated to liposomes (Tao et al. 2012),
335variable heavy-chain domain of camel homodimeric antibodies (VHH) (Li et
336al., 2012) for receptor-homing peptides obtained from phage display
337screening (Maggie et al. 2010; Malcor et al. 2012). To gather all published
338information related to peptides with activity to cross the BBB, Van Dorpe and
339collaborators designed a peptide database to organize scattered information
340(Van et al. 2012) (<http://brainpeps.ugent.be>). The main approaches to protein-
341guided BBB delivery of therapeutic nanoparticles are summarized in Figure 4.

342

3436. BBB-crossing for the treatment of CNS diseases.

344 Among CNS diseases, only three are currently treated with drugs that
345naturally cross the BBB, namely epilepsy, chronic pain and psychiatric
346disorders (Ghose et al. 1999). For degenerative diseases, vascular diseases,
347trauma aftermaths, viral infections and congenital diseases occurring in the
348CNS, there is a pushing need to develop BBB-crossing strategies for drug
349delivery, preferentially based on non-viral carriers (Table 2). The most
350representative examples of how BBB-crossing is addressed in these
351conditions are discussed in the next sections.

3536.1. Neurodegenerative disorders

354 Therapeutic approaches to neurodegenerative diseases are
355concentrating most of the efforts on the design of therapeutic compounds able
356to cross the BBB. For Parkinson's disease, the first drug used clinically was
357the dopamine precursor L-Dopa, that contrarily to dopamine itself, crosses the
358BBB by using a large amino acid transporter (Wade and Katzman 1975). On
359the other hand, in a Trojan Horse approach, Pardridge's group normalized
360striatal tyrosine hydroxylase levels and reversed functional signs in a
361Parkinson model. A tyrosine hydroxylase gene empowered by a nervous
362system-specific promoter was injected, carried by pegylated liposomes
363decorated with OX26 antibody against TfR (Zhang et al. 2003; Zhang et al.
3642004a). The team was also successful entering erythropoietin (Zhou et al.
3652011b) and glial derived neurotrophic factor (GDNF) (Fu et al. 2010a) by
366joining these therapeutic proteins to mice anti-TfR antibodies, and
367subsequently reaching clear neuroprotective effects.

368 Regarding Alzheimer, again, by means of this anti-TfR antibody as BBB
369transporter and by fusion to an anti-Abeta amyloid antibody, the levels of beta
370amyloid peptide were dramatically reduced (Zhou et al. 2011a). In this
371context, Genentech is developing a lower affinity variant of anti-TfR antibody
372(that favors release from the BBB towards the CNS) fused to an antibody
373against the enzyme BACE1, involved in amyloidal plaque formation. When the
374bifunctional molecule is applied systemically, a decrease of 47 % in plaques
375was observed in mouse models (Yu et al. 2011). Interestingly, the fusion of a
376monovalent sFab of an anti-TfR antibody to an anti-Abeta antibody mediated
377effective uptake transcytosis and TfR recycling, while the presence of two Fab
378fragments on the anti-Abeta antibody resulted in uptake followed by trafficking
379to lysosomes and an associated reduction in TfR levels (Niewoehner et al,
3802014). This approach exhibited enhanced *in vivo* targeting of Abeta plaques
381after i.v. administration. Nerve growth factor (NGF) fused to an anti-TfR
382antibody has also been used successfully to prevent neuronal degeneration
383when applied intravenously in a Huntington disease model (Kordower et al.
3841994). In a similar context, a poly(mannitol-co-PEI) gene transporter modified

385with a rabies virus glycoprotein is able to ameliorates Alzheimer symptoms by
386transporting a therapeutic RNAi (Park, 2015). Alternatively, the intranasal
387route to the CNS (Hanson and Frey 2008), through the olfactory via and
388trigeminal nerve has been largely explored to introduce important factors in
389neurogenesis and memory such as NGF (De et al. 2005), insulin-like growth
390factor 1 (IGF- I) (Liu et al. 2004), fibroblast growth factor 2 (FGF-2) (Jin et al.
3912003), insulin (Benedict et al. 2004), interferon beta (IFN beta (Ross et al.
3922004) and the octapeptide NAP (Matsuoka et al. 2008) which is currently in
393Phase II clinical trials in patients with incipient Alzheimer 's disease (Gozes et
394al. 2009).

395

3966.2 *Brain tumors*

397 Diverse BBB-crossing anti-tumor vectors are under development in
398both pre-clinical and clinical phases, empowered by a spectrum of BBB-
399crossing tags. Angiochem Inc. entered into Phase I clinical trials a product
400(ANG1005) that uses the peptide Angiopep-2, capable of driving the cargo
401paclitaxel by transcytosis through the BBB by using the LDL receptor LRP- 1.
402This conjugate showed previously intracranial tumor regression in murine
403models when administered i.v. (Bichat 2008). Melanotransferrin associated
404with doxorubicin increased the survival in mice with intracranial tumors
405(Gabathuler 2005; Karkan et al. 2008). Albumin is being used at University of
406California, San Francisco (UCSF), in a Phase I clinical trial as a carrier of
407paclitaxel (nab- paclitaxel) to treat brain and CNS tumors (Chien et al. 2009)
408(it is already in the market for breast cancer). Targeting the transmembrane
409protein TMEM30A, the ligand FC5 (discovered by phage display, a single
410domain antibody – sdAb-), drives liposomes though the BBB to release
411doxorubicin into CNS (Gabathuler 2010a). On the other hand, by taking a
412Trojan Horse strategy based on pegylated immunoliposomes targeted to TfR
413(Boado et al. 2007), the delivery of shRNAs expression vectors against the
414epidermal growth factor receptor (EGFR) increased the survival in mice with
415intracranial tumors (Boado 2007; Pardridge 2004; Zhang et al. 2004b).
416Doxorubicin ferried by polysorbate-coated polymer nanoparticles promoted
417long-term glioblastoma remission in rats, probably by an unspecific BBB
418crossing (Steiniger et al. 2004), and a polycefin polymer variant that

419specifically targets human brain, which associated to antiangiogenic
420oligonucleotides inhibits tumor angiogenesis and improves animal survival
421(Ljubimova et al. 2008).

422 On the other hand, despite no direct CNS targeting, it has been
423possible to increase the intracranial levels of anticancer 3'5'-dioctanoyl-5-
424fluoro-2'deoxyuridine (DO-FUdR), by incorporating it into a solid lipid
425nanoparticle (Wang et al. 2002). Furthermore, when administered
426systemically, nude phosphorothioate oligonucleotides against protein kinase C
427alpha, also reduced intracranial glioblastoma tumor size and doubled mice
428survival time (Yazaki et al. 1996). On the basis of these results, a phase II
429clinical trial has been completed ([http://www.clinicaltrials.gov/ct2/results?](http://www.clinicaltrials.gov/ct2/results?term=pkc-alpha)
430term=pkc-alpha). In a more recent example, an intravenously injected cell
431penetrating peptide (LNP) decorating a polylysine-PEG gene vector extended
432the median survival time of glioma-bearing mice (Yao et al. 2014).

433

4346.3 *Pain*

435 Anti-nociception is usually achieved by methylation (Hansen, Jr. et al.
4361992) or glycosylation (Polt et al. 1994) of active molecules to stimulate their
437penetrability into the CNS. On the other hand, coupling human serum albumin
438to an anti-TfR permits the transport of loperamide into the CNS for anti-
439nociception effects (Ulbrich et al. 2009). The same drug is delivered into the
440CNS by injecting i.v. a poly(lactic-co-glycolic) acid (PLGA) nanoparticle,
441derivatized with the peptide H₂N-Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O-β-
442D-Glucose)-CONH₂ (g7) (Tosi et al. 2007). The analgesic dalarginine joined to a
443cationic cell-penetrating peptide (Syn-B) increases brain uptake in two orders
444of magnitude. This peptide crosses the BBB using a nonspecific route, that is,
445without association with a receptor (Rousselle et al. 2003). Other
446polyarginine-based peptides as CNS transporters are in preclinical phases
447(Gabathuler 2010a).

448

4496.4. *Ischemia*

450 Sequelae of cerebral ischemia can be lessened by CNS deliver of
451brain-derived neurotrophic factor (BDNF) (Wu and Pardridge 1999; Zhang
452and Pardridge 2001), fibroblast growth factor (FGF-2) (Song et al. 2002),

453inhibitor of caspase-3 (Yemisci et al, 2014), vasoactive intestinal peptide (VIP)
454(Bickel et al. 1993; Wu and Pardridge 1996) and erythropoietin (EPO) (Fu et
455al. 2011) linked to an anti-TfR antibody. The nerve growth factor (NGF) gene
456has been introduced into the CNS while inside lipoplexes decorated with the
457TfR natural ligand, transferrin (da Cruz et al. 2005). The cell penetrating Tat
458peptide has also proven to carry efficiently N-methyl D-aspartate receptor
459subtype 2B (NR2B) domain (Aarts et al. 2002), B-cell lymphoma-extra large
460protein (Bcl-X_L) (Kilic et al. 2002), glial cell-derived neurotrophic factor (GDNF)
461(Kilic et al. 2003) and c-Jun domain (Borsello et al. 2003), to protect neurons
462in brain infarct models. On the other side, sniffing insulin-like growth factor
463(IGF-1) (Liu et al. 2004) and EPO (Yu et al. 2005) protects brain against
464stroke in animal models (Hanson and Frey 2008). Modular protein/DNA
465nanoparticles have been shown to induce biologically relevant transgenic
466protein levels and therapeutic effects after acute excitotoxic injuries when
467injected intracerebrally (Negro-Demontel, et al., 2014; Peluffo et al. 2003;
468Peluffo et al. 2006; Peluffo et al. 2011). The addition of CNS targeting
469domains to these particles may enable intravenous delivery retaining its
470neuroprotective potential.

471

4726.5 *Infectious diseases*

473 CNS infectious diseases have also been treated *in vivo* using different
474approaches. By administering i.v. siRNA into Japanese encephalitis virus-
475infected mice, Manjunath and cols. afforded specific viral gene silencing and
476protection. The siRNA carrier was a two-domain peptide formed by nine
477arginines (R9) and a peptide derived from rabies virus glycoprotein (RVG)
478(Kumar et al. 2007). On the other hand, the brain levels of different anti HIV
479drugs have been increased several folds through association with liposomes
480(foscarnet, (Dusserre et al. 1995)), micelles (zidovudine, lamivudine,
481nelfinavir, (Spitzenberger et al. 2007)) and the Tat protein (ritonavir, (Rao et al.
4822009)). Furthermore, second stage African trypanosomiasis was treated
483intravenously in a mouse model by conjugating the active water-soluble drug
484to liposomes using polysorbate 80 as surfactant (Olbrich et al. 2002).

485

4866.6. *Other conditions*

487 Other diseases in which the BBB crossing has been successfully
488 achieved are Hurler's Syndrome (mucopolysaccharidosis), using the mouse
489 anti-TfR antibody associated to a liposome with beta-glucuronidase gene
490 (Zhang et al. 2008) or fused to the alpha-L-iduronidase enzyme (Boado et
491 al. 2008). A cell-penetrating Tat peptide improves the beta-glucuronidase
492 biodistribution when organized as a single chain fusion protein (Xia et al.
493 2001). Narcolepsy has also been treated with good results with nasal
494 hypocretin I (Hanson and Lobner 2004).

495

496

497. **Administration routes**

498 The intravenous administration of functionalized nanoparticles is the
499 most used therapeutic route. However, in some cases, patient compliance is
500 not easy to achieve, and alternative administration routes need to be
501 explored. In fact, there are standardized methods for drug delivery by osmotic
502 disruption (Kroll and Neuwelt 1998; Yang et al. 2011), by local delivery placing
503 polymer wafers after tumor excision (Balossier et al. 2010), by convection-
504 enhanced delivery (White et al. 2012a; White et al. 2012b) or by intranasal
505 administration (Grassin-Delye et al. 2012; Tsai 2012; Wolf et al. 2012; Zhu et
506 al. 2012) (Figure 1 A). Some of these treatments are still highly invasive and
507 are only addressed to high grade glioma patients. In the milder intranasal
508 delivery, the drug is being accumulated in the olfactory bulb and then diffusing
509 inside the brain. This approach has been proven to be quite effective in the
510 treatment of various disease models, acting through the olfactory pathway
511 and trigeminal nerve (Born et al. 2002; Hanson and Frey 2008). Regarding
512 gene therapy, only 1.9 % of current clinical trials are performed on the CNS,
513 and almost all of them are applied by intracranial injection or performed ex
514 vivo (Ginn et al. 2013), pointing to the importance of the delivery of BBB-
515 crossing gene therapy vectors.

516

517. **Conclusions and future prospects**

518 Numerous examples of basic research and ongoing clinical trials illustrate
519 how proteins can be engineered to overcome the complexity of both BBB and
520 BSCB in drug delivery contexts. In this regard, a few CNS diseases are

521already treated with protein-based targeted drugs, and much more are
522expected to be released for use in the next future. Hopefully, and based on
523current insights on the engineering of protein self-assembling, functional
524proteins would be desirably adapted as building blocks of nanosized entities,
525acting at the same time as BBB crossers, targeting agents and drug carriers.
526Although the fully de novo design of such protein-based artificial viruses is in
527its infancy, the accumulation of data about the physiology of the CNS and of
528relevant cell receptors, the widening spectrum of drugs potentially useful in
529CNS therapies and the exploration of alternative routes for administration on
530the bases of result from the use of natural viruses envisage the generation of
531these sophisticated vehicles as a forthcoming routine strategy.

532

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547

548**Legends:**

549**Figure 1.** Anatomical basis of the BBB. Boundaries of the CNS tissue
550contacting the blood vessels, meninges and the cerebrospinal fluid are
551depicted (A), and also alternative routes for administration of substances to
552the CNS to bypass the BBB. The intimate relationship between ECs,
553continuous basement membrane, astrocytes, pericytes and perivascular
554macrophages contributing to various degrees to the BBB formation and

555maintenance can be observed (B). Moreover, ependimocytes of the choroid
556plexus produce the cerebrospinal fluid and conform, in addition, the Blood
557Cerebrospinal Fluid Barrier (BCFB) (C).

558

559**Figure 2.** Main barriers and transport mechanisms of the BBB. Physical
560barriers as endothelial cell membranes or intercellular tight junctions are the
561principal obstacles to overcome for polar macromolecules to enter the CNS
562(left). Moreover, intracellular and extracellular enzymes, basal membrane and
563astrocyte endfeet can also constitute additional barriers. Endogenous protein
564mediated selective transport mechanisms for small polar substances and
565macromolecules are the responsible for the communication of the CNS with
566the blood flow (right). These can be exploited for targeted delivery of different
567types of nanocomplexes.

568

569**Figure 3.** AAV9 administration routes and transduction efficiencies. Different
570results have been obtained when AAV9 where administered by i.v. or intra-
571thecal delivery, but also in postnatal or adult animals, and importantly in mice
572or in non-human primates. While i.v. delivery efficiently transduce neurons and
573astrocytes in postnatal and adult mice, very low efficiency and mainly
574astrocyte transduction was observed in non-human primates. Moreover,
575intrathecal delivery into the Cisterna Magna resulted in the widest
576transduction in non-human primates.

577

578**Figure 4.** Receptor-mediated approaches used in Nanomedicine to cross the
579BBB. Different types of proteins (including antibodies) showing specific
580binding to BBB transporters and cell surface receptors that are relevant to
581transcytosis are used to functionalize nanoparticles (NPs). Cell-penetrating
582peptides carrying therapeutic proteins are also depicted. More details and
583specific examples are given in Table 1.

584

585

586

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

587

Method	Target	Ligand and references	Application and NP size reference
Therapeutic proteins conjugated to mAbs raised against insulin and transferrin receptors	Transferrin receptor Insulin receptor	Carboxy terminus of the IgG heavy chain(mAb) against the mouse transferrin 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	Erythropoietin fused to the mAb to treat Stroke (Fu et al. 2011) Insulin or an anti-insulin receptor mAbs were covalently coupled to the Human serum albumin NP (Zensi et al. 2010a)
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)
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)

Transferrin receptor	Lactoferrin	Pegylated NPS conjugated with lactoferrin used for imaging diagnostic purposes (Qiao et al. 2012a)	Fe ₃ O ₄	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

588

589mAbs: monoclonal antibodies
590LDLR: low density lipoprotein receptor
591Apo: apolipoprotein
592NP: nanoparticle
593ND: not determined
594THR: tri-peptide motif (thre-his-arg)

595Table 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 against TfR.	(Zhang et al. 2003; Zhang et al. 2011b)
	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)
Huntington 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 receptor	Drug associated with Melanotransferrin	(Karkan et al. 2008)
	Paclitaxel	ND	Drug conjugated to Albumin	(Chien et al. 2009)
		TMEM30A transmembrane protein		
	Doxorubicin		Liposomes decorated with FC5 ligand	(Gabathuler 2010a)

Anti-nociception	shRNAs against EGFR	Insuline Receptor / Transferrine receptor LDL receptor via	Pegylated immunoliposomes associated to TfR ab and Insulin receptor Ab.	(Boado 2007; Pardridge 2004)
	Doxorubicin Oligonucleotides against protein kinase C alpha	ApoB/E enrichment ND	Drug bound to polysorbate-coated polymer Nude oligonucleotide administration	(Steiniger et al. 2004) (Yazaki et al. 1996)
	Loperamide	TfR Possible adsorption- mediated	Human serum albumin coupled to TfR ab. PLGA nanoparticle derivatized with a glycosylated heptapeptide	(Ulbrich et al. 2009) (Tosi et al. 2007)
	Loperamide	endocytosis		
	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 ischemia	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-XI	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)
		LDL receptor via		
	Diminazenediaceturate	Apo E enrichment	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)

596ND: not determined
597PLGA: Poly(lactic-co-glycolic) acid
598TfR: Transferrin receptor

599

600

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601

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