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Review

From naturally-occurring neurotoxic agents to CNS shuttles for drug delivery

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ABSTRACT

Central nervous system (CNS) diseases are hard to diagnose and therapeutically target due to the blood brain barrier (BBB), which prevents most drugs from reaching their sites of action within the CNS. Brain drug delivery systems were conceived to bypass the BBB and were derived from anatomical and functional analysis of the BBB; this analysis led researchers to take advantage of brain endothelial membrane physiology to allow drug access across the BBB. Both receptors and carriers can be used to transport endogenous and exogenous substances into the CNS. Combining a drug with substances that take advantage of these internalization mechanisms is a widely exploited strategy for drug delivery because it is an indirect method that overcomes the BBB in a non-invasive way and is therefore less dangerous and costly than invasive methods.

Neurotoxins, among other naturally-occurring substances, may be used as drug carriers to specifically target the CNS. This review covers the current delivery systems that take advantage of the non-toxic components of neurotoxins to overcome the BBB and reach the CNS. We hope to give insights to researchers toward developing new delivery systems that exploit the positive features of substances usually regarded as natural hazards.

Contents

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1. Introduction

Central nervous system (CNS) disorders are often difficult to diagnose and treat because the blood brain barrier (BBB) denies most drugs access to the CNS. The BBB is a highly complex cerebrovascular system comprised of approximately 100 billion capillaries, whose endothelial cells are strictly joined with tight junctions (Brightman et al., 1969) and surrounded by astrocytes, pericytes, and macrophages, found throughout the dense and complex barrier that protects the CNS. The BBB protects the CNS from outside intrusion but also prevents entry of therapeutic drugs to the brain; more than 98% of drugs with a molecular weight less than 400 Da and all those with higher weights do not cross the BBB (Pardridge, 2003, 2005). The treatment of diseases such as migraine, insomnia, affective disorders, pain, and epilepsy is based on the use of small, well-known molecules; on the other hand, the more devastating diseases of the CNS, such as brain tumors, stroke, and neurodegenerative disorders, do not have cures or treatments available because the large-molecule drugs that prevent proliferation of these diseases cannot pass the BBB. Most new drugs developed to treat CNS disorders are abandoned in phase I or II clinical trials, after costly development, because of poor access to nervous tissue (Khrestchatisky and Tokay, 2014). To treat these CNS diseases, new delivery strategies must be developed to allow these large molecules to reach the CNS.

Multiple approaches are currently used to bypass the BBB and deliver therapeutic drugs to the brain (reviewed by Alam et al. (2010) and Gabathuler (2010)). Invasive approaches include physical techniques, such as intra-cerebroventricular infusion, convection-enhanced delivery, and polymer or microchip systems, which, after implantation, directly release therapeutics into the CNS and disrupt the BBB by opening the tight junctions between the endothelial cells of the brain capillaries. Invasive techniques have significant limitations, such as low diffusion of the drug into the brain parenchyma or the requirement for neurosurgery. Invasive approaches are painful, dangerous, non-patient friendly, and expensive, since they require qualified medical staff and appropriate instrumentation and are not always applicable (Alam et al., 2010; Gabathuler, 2010).

Non-invasive approaches to carry drugs to the CNS include nasal administration; the direct pathway from the nose to brain bypasses the BBB. Drug delivery from the nose to the CNS occurs via the olfactory epithelium and may involve paracellular, transcellular, and/or neuronal transport (Pires et al., 2009; van Woensel et al., 2013). Several delivery systems, such as micro- and nanoparticles, were studied to improve CNS uptake of drugs by nasal administration, as an alternative to the oral and parenteral routes, which increased the amounts of drug in the CNS compared to not formulated drug (Dalpiaz et al., 2008; Gavini et al., 2011, 2013; Josea et al., 2013; Kumar et al., 2013; Fazil et al., 2012).

Indirect approaches can be classified as follows:

- Pharmaceutical methods, where the physico-chemical characteristics of the drug are adapted to ensure that the drug crosses the BBB. These methods are based on direct structural modifications of the drug (prodrugs) or by the use of additional system formulations, such as liposomes or nanoparticles (Witt et al., 2000; Tiwari et al., 2006).
- Barrier modification methods, which change the structure of the BBB by temporarily increasing its permeability by osmotic or biochemical opening or using ultrasound or electromagnetic modulators (Doolittle et al., 2000; Huang et al., 2012; Plank et al., 2011).
- Physiological methods, which are interesting, versatile strategies that exploit the transporters, receptors, or enzymes present in the brain endothelium to allow drugs to bypass the BBB. Physiological methods can be used with different stratagems to deliver drugs of different natures, selectively targeting the CNS in a safe way without invasiveness. Exploiting structures physiologically (Fig. 1) means to use endogenous mechanisms that allow substances to pass undisturbed, regardless of their chemical–physical characteristics.

Currently, multiple systems have been developed, such as molecular Trojan horses, chimeric peptides, cationic proteins, prodrugs, and the use of vectors as transport/carrier systems.

Molecular Trojan horses use genetically-engineered proteins to carry drugs to the BBB surface; the entire complex is transported by a receptor-mediated mechanism (Pardridge, 2006).

The chimeric peptide system is obtained by covalent bonding of the pharmaceutical substance to a peptide or protein vector that can traverse the BBB by absorptive- or receptor-mediated transcytosis (Prokai et al., 2003).

Cationic protein systems favor the penetration of protein-based drugs through the BBB by electrostatic interactions between the cationic protein and the negative functional groups present in the barrier, resulting in internalization by transcellular, adsorptive-mediated endocytosis pathways (Bradbury et al., 2000).

Prodrugs are obtained by coupling a drug with a site-specific vector moiety to obtain a system without intrinsic activity that must undergo a chemical reaction to regenerate the active agent *in vivo*. This is often possible by the action of target-specific enzymes present on the BBB surface.

Vector-mediated delivery employs drugs conjugated to substances that can normally cross the BBB.

Neurotoxins are chemical or biological substances that target the CNS, by different mechanisms, and cause neurodegenerative disorders (Salinas et al., 2010) by affecting the transmission of chemical signals between neurons. Neurotoxins disrupt the signaling that allows neurons to communicate effectively by blocking ion channels or inhibiting the signaling molecules (or their release) that propagate the action potential from one neuron to the next, sometimes generating false signals; neurotoxins can also simply damage or destroy neurons. Neurotoxins can have pre- or post-synaptic actions and can be classified as acting on ion channels, neurotransmitter receptors, or enzymatic mechanisms (Tipton and Dajas, 1994); some neurotoxins are being developed as vectors for vector-associated drugs to cross the BBB and target the CNS.

The first use of neurotoxic derivatives in drug delivery systems dates back to the year 2000, when botulinum toxin was used to

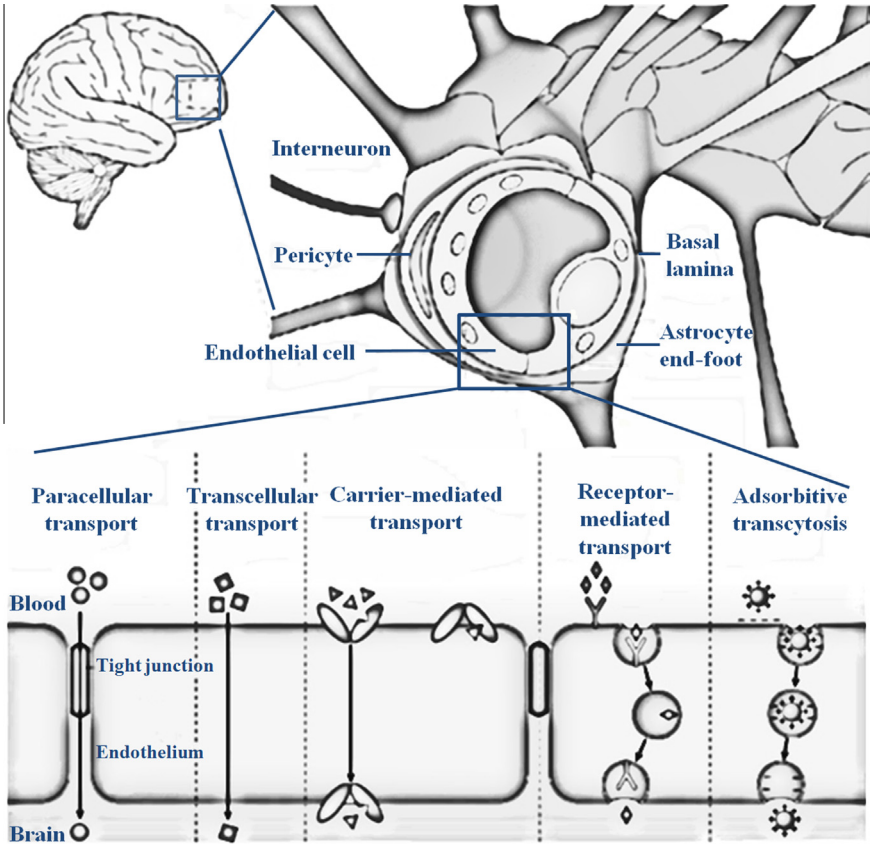

Fig. 1. Schematic representation of the BBB and main transport mechanisms present in the brain endothelium.

deliver drug (Chaddock et al., 2000), followed by tetanus toxin (Bordet et al., 2001).

We investigated studies on the use of naturally-occurring neurotoxins as potential shuttles in brain delivery systems, classified by origin: viral (Table 1), bacterial (Table 2), and animal (Table 3).

2. Shuttles from viruses

2.1. Herpes virus

Herpes simplex viruses 1 and 2 (HSV1 and HSV2) and varicella-zoster virus (VZV) are human neurotropic double-stranded DNA viruses; from outside to inside, they are composed of pericapsid or envelope, tegument, capsid, and core. The pericapsid is the external coating, a lipid bilayer which contains several viral glycoproteins responsible for binding to specific cellular receptors and penetration of the virus into the host cytoplasm. The tegument is composed of a protein matrix responsible for virion assembly, shut-off of host proteins, and induction of viral genes. The capsid has a symmetrical icosahedral structure, is surrounded by the tegument, and contains the core, which contains the viral genome. After primary infection, HSV1, HSV2, and VZV can persist in a state of latency, primarily in the dorsal root ganglia and trigeminal nerve. In the presence of particular stimuli (environmental or subjective), the virus can reactivate and migrate along the nerve endings to reach the primary sites of infection, such as the cutaneous areas and mucous membranes (Steiner et al., 2007).

The 19-residue peptide gH625 is derived from the gH fusion glycoprotein (from aa 625 to aa 644) of HSV1; it facilitates viral particle envelope fusion with the host cell plasma membrane, working as a membrane-perturbing domain. Similar to CPPs (Cell-Penetrating Peptides), gH625 can transport cargo molecules across cell membranes through a translocation mechanism. gH625 has been used for functionalization of nanoparticles (NPs), quantum dots, dendrimers, and liposomes to promote and study their uptake into cells (Falanga et al., 2013; Smaldone et al., 2013). In the context of brain delivery systems, gH625 was covalently linked to an aminated polystyrene surface (gH625-NPs) (Guarnieri et al., 2013) and different peptide densities were used to characterize and investigate the intracellular trafficking of gH625-NPs; a surface density of peptide of about 3, 4, and 6 peptides/nm^2 were used for 25%, 35%, and 50% functionalized NPs, respectively (Guarnieri et al., 2013). Since NPs with 35% functionalization did not induce particle aggregation, were stable in aqueous medium, and were better able to go inside cells via a random walk behavior, these were used for *in vitro* BBB-crossing studies. gH625-NPs, in BBB models based on an immortalized mouse cerebral endothelial cell line (bEnd3), had higher uptake than non-functionalized nanoparticles and were better able to escape endocytosis entrapment. Thus, gH625-NPs can increase drug entry into the CNS, despite the lack of site-specificity; however, because the peptide has a membrane-perturbing domain, it could act on all cell membranes, leading to peripheral and unwanted side effects. The same authors reported that functionalization with the peptide allows the entry of quantum dots and liposomes into HeLa cells, suggesting additional functionalization of nanoparticles with molecules that selectively recognize the cerebral endothelium.

2.2. Rabies virus

The rabies virus is a neurotropic, rod- or bullet-shaped, single-stranded, negative-sense, unsegmented, and enveloped RNA virus

Table 1
CNS delivery systems based on viral neurotoxins. NPs: nanoparticles; LSPCs: siRNA–liposome–peptide complexes; i.v.: intravenous; NC: nanocarrier; TMC: trimethylated chitosan; ITZ: itraconazole.

VIRUS						
Species	Brain targeting moiety	Target action	Brain delivery systems	Drugs	Biologic properties	References
Herpes virus simplex	gH625	Cell membrane	gH625-NPs	–	In *in vitro* BBB models, higher uptake compared to the same particles not peptide functionalized	Guarnieri et al. (2013)
Rabies virus	RVG	nAchRs	RVG-9R/oligonucleotide complex	siRNA	Ability to selectively reach neuronal cells, to transduce siRNA inside and to permit silencing effect both in *in vitro* than in *in vivo* experiments	Kumar et al. (2007)
			RVG-9R/oligonucleotide complex	P137 RNA	After intravenous injection in rats, ability to prevent nigrostriatal dopaminergic damage, indicating that RNA cargo was able to cross the BBB and reach its target in CNS	Kuan et al. (2012)
			RVG-9R/gene complex	pDNA	Ability to transfect neuronal cell models (Neuro2A) more efficiently compared to a common transfection reagent. Peripheral injection in mice leads to gene expression	Gong et al. (2012)
			LSPCs	siRNA	Ability to decrease prion infection in prion infected N2a and NCerP cell culture models; brain gene silencing after i.v. injection in FVB mice	Pulford et al. (2010)
			Pluronic-based NC RVG29-conjugated	β-galactosidase	Ability to reach brain after i.v. administration in nude mice, with retention of drug bioactivity	Kim et al. (2013)
			PAMAM-PEG-RVG29 NPs	DNA	Capacity to accumulate into the brain and to regulate gene expression after i.v. injection in mice	Liu et al. (2009)
			NPs SSPEI-RVG29	miRNA	Capacity to achieve the brain in *in vivo* studies	Hwang et al. (2011)
			BPEI-SS-PEG-RVG29	pDNA	In *in vivo* test, evidence of the central role of RVG to reach CNS	Son et al. (2011)
			RVG29-PEG-TMC-NPs-RVG29	siRNA ITZ	Accumulation in the brain of siRNA/TMC Enhancement of NPs intracellular delivery in neuronal cells. Significant accumulation of ITZ into the brain	Gao et al. (2014) Chen et al. (2011)
	RDP		RDP-9R peptides conjugated	β-Gal, Luc, BDNF	Efficient transport of the 3 biologically active proteins into the mouse CNS after i.v. injection	Fu et al. (2012a)
			RDP/plasmid complexes	Lac Z, BDNF	Efficient brain-targeting of plasmid DNA after peripheral injection. The drug activity was ensured	Fu et al. (2013)

Table 2
CNS delivery systems based on bacterial neurotoxins. NPs: nanoparticles; i.c.v.: intracerebroventricular.

BACTERIA						
Species	Brain targeting moiety	Target action	Brain delivery systems	Drugs	Biologic properties	References
Vibrio Cholerae	CTB	GM1 ganglioside.	CTB-conjugated	saporin	Ability to reach the CNS. The drug maintained its activity	Ohara et al. (2005)
			CTB-conjugated	myelin basic protein	Ability to cross the BBB and retinal barrier. The drug has preserved its activity.	Kohli et al. (2014)
			CTB-conjugated	NGF	Capability to allows NGF reaching the brain only by olfactory nerve, after nasal administration. Capability to improve NGF curative properties in *in vivo* Parkinson model	Zhang et al. (2008)
	Delta G	Tight junctions	DeltaG fragment co-administrated with drugs	methotrexate, paclitaxel	In *in vivo* experiment, improvement of brain drug distribution	Menon et al. (2005)
Corynebacterium diphteria	Cross-reacting material 197 (CRM197)	Diphteria toxin receptor (DTR)	CRM197-conjugated	horseradish peroxidase	Ability to selectively target CNS both in *in vitro* BBB models than after intravascular injection in guinea pigs. Safe and effective brain drug delivery carrier for proteins	Gaillard et al. (2005)
			NPs CRM197-PBCA	zidovudine	Ability to cross *in vitro* BBB models and to carry zidovudine through the barrier	Kuo and Chung (2012)
			NPs CRM197-PEG-PEI	siRNA	Tumor therapeutic efficacy in glioblastoma model mice	Höbel et al. (2011)
Clostridium botulinum	Botulinum neurotoxin A (BoNT-A)	Presynaptic cholinergic nerve cells at the peripheral neuromuscular junctions	BoNT-A_conjugated	10-kDa dextran	Internalization in primary cultures of spinal cord	Zhang et al. (2009)
			BoNT/A		Capability to reach cellular cytoplasm suggesting a potential use as a system to address multiple medical and biodefense needs	Vazquez-Cintron et al. (2014)
	Botulinum neurotoxin D BoNT/D		BoNT/D_conjugated	protein models	Ability to carry protein models, in a functional active form, into neuronal cytosol.	Bade et al. (2004)
Clostridium tetani	Non-toxic C-fragment of tetanus toxin (TTC)	Motorneurons of neuromuscular junction	TTC-conjugated	glucose oxidase	Capability to be retrogradely transported from NMJ to the spinal-cord motoneuron after i.m. injection in mice	Beaude et al. (1990)
			TTC-conjugated	human Cu/Zn superoxide dismutase	Enhancement of proteins CNS bioavailability after i.c.v injection allowing the achievement of target neurons in the brain parenchyma through the cerebrospinal fluid	Benn et al. (2005)
			TTC-conjugated	GDNF	Enhancement of drug accumulation and neuroprotection in spinal motorneurons after i.m. injection to mice	Larsen et al. (2006)
			TTC-conjugated	Cardiotrophin-1	In *vitro* motoneuron survival	Bordet et al. (2001)
			TTC-conjugated	Bcl-xcL, IGF1, BDNF	Specific internalization and retrograde neuron transport with retention of the biological activity of the peptides	Carlton et al. (2008); Payne et al. (2006); Roux et al. (2006)
Clostriudium perfringens	Epsilon toxin precursor (ETXp)	Unknown receptor in the BBB and myelinated brain regions	ETXp-conjugated	bleomycin	Bleomycin effectively reach the CNS. Efficiency of the system was enhanced using photochemical internalization. Human application limited because of ETXp toxicity	Hirschberg et al. (2009)

Table 3
CNS delivery systems based on animal neurotoxins. NPs: nanoparticles; i.v.: intravenous; TRAIL (tumor necrosis factor-related apoptosis-inducing ligand).

ANIMALS						
Species	Brain targeting moiety	Target action	Brain delivery systems	Drugs	Biologic properties	References
Bungarus candidus snake	Candoxin (CDX)	nAchRs	PEG-PLA-CDX micelles	paclitaxel	Ability to reach the brain compared to NPs not functionalized	Zhan et al. (2011)
			PEG-PLA-CDX micelles and RGD-PEG-PEI/ pORF-hTRAIL NPs	paclitaxel, TRAIL	Synergistic effect of the anticancer drugs transported by the co-administration of the two brain delivery systems	Zhan et al. (2012)
Ophiophagus Hannah	Synthetic peptide from toxin B (KC2S)		KC2S-PEG-PLA micelles	paclitaxel	Enhancement of intracranial drug delivery after i.v. treatment	Zhan et al. (2010)
Crotalus molossus molossus	Polylysine-molossin	Unknown	Polylysine-molossin/ oligonucleotide complexes	DNA	Ability to increases DNA transfection efficiency in nerve cells but also in non-nervous cells and tissues	Collins et al. (2003)
Leiurus quinquestriatus hebraeusis	Chlorotoxin (ClTX)	Lipid raft-anchored complex containing matrix metalloproteinase-2 (MMP-2)	NPs-copolymer coated-ClTX-Cy5.5 and NPs-ClTX	DNA	Selective targeting of *in vivo* brain tumors	Fu et al. (2012b)
			Liposomes-ClTX	doxorubicin	Enhancement of liposomes uptake by glioma and endothelial cell lines. Accumulation in brain tumors of animal models	Xiang et al. (2011)
			Liposomes-ClTX	levodopa	*In vitro* uptake enhancement of brain microvascular endothelial cells	Xiang et al. (2012)
			Liposomes-ClTX	miRNA, siRNA	Capability to reach intracranial tumor after i.v. administration	Costa et al. (2013)
			Iron oxide NPs coated with PEG-chitosan-ClTX	Supermagnetic iron oxide, Cy5.5	Ability to cross the BBB and specifically target brain tumors in a genetically engineered mouse model	Veiseh et al. (2009)
	TM-601, ClTX synthetic version		131-I-TM-601	131-I	Due to its ability as a brain targeting moiety, started a Phase I clinical trials in 2006; the aim was to evaluate the ability of intravenously administered 131-I-labeled TM-60 for tumor-specific localization	https://clinicaltrials.gov/show/NCT00379132.
Apis millifera	ApOO	Low conductance Ca2+ -activated K+ channels	ApOO	–	Ability to pass through an *in vitro* BBB model	Gomara et al. (2003)
	Melittin	Lipid membrane. Ability to induce pore formation	pHgMelbHK10/gene polyplexes	pDNA	Inability to discriminate between HeLa or PC12 cell. Ability to target the brain only after invasive intraventricular administration	Schellinger et al. (2013)

(Rupprecht, 1996). After peripheral infection, rabies virus reaches the CNS through retrograde transport (Rupprecht, 1996; Klingen et al., 2008). RVG29 is a 29-residue peptide derived from a rabies virus glycoprotein that binds specifically to α7 nicotinic acetylcholine receptors (nAchRs), which are highly expressed in the CNS. Kumar et al. (2007) were the first to demonstrate the use of RVG29 as a ligand for safe, non-invasive CNS drug targeting. At first, the authors synthesized a chimeric peptide, RVG-9R, by adding 9 arginine residues to RVG29 to favor electrostatic interaction with an oligonucleotide; subsequently, RVG-9R peptide was electrostatically complexed with a siRNA oligonucleotide (RVG-9R/siRNA). The resulting unprotected complex selectively interacted with α7 nicotinic receptors, thus ensuring a selective delivery to neuronal cells, allowing siRNA transduction inside cells and the silencing effect in both *in vitro* and *in vivo* experiments. Kumar et al. (2007) used flow cytometry to analyze the possible effects of the chimeric peptide on peripheral tissues and concluded that the cargo delivery was restricted to the CNS; toxicity was assessed *in vitro* by determining cell viability and *in vivo* by evaluating the immune stimulating effect. The RVG-9R/siRNA complex appeared safe but had limited serum stability (8 h). To overcome the limited serum stability, the authors suggested using RVG29 as a brain targeting ligand in: (1) siRNA-encapsulated nanoparticles to protect the oligonucleotide, enhance delivery, and decrease its requirement for effective gene silencing; (2) nanoparticles encapsulating other gene or small-molecule drugs; and (3) systems obtained by direct conjugation with siRNA, instead of electrostatic complexation between an oligonucleotide and the 9 arginine residues of RVG peptide.

A complex composed of RVG-9R and p137 RNA, a viral noncoding RNA that prevents dopaminergic neuronal death (Kuan et al., 2012), was proposed as a potential treatment for Parkinson's disease. The peptide facilitated the delivery of RNA across the BBB. After transvascular administration in rats, the conjugate was neuroprotective against damage to nigrostriatal dopaminergic cells, indicating that the RNA cargo crossed the BBB and reached its CNS target by interaction of the peptide with the α3/α5 nicotinic receptors richly expressed by CNS cells; however, Kuan and collaborators did not account for the pharmacokinetic parameters of the RVG-9R–p137 conjugate and thus no plasmatic half-life or stability data were reported. The RVG peptide can target siRNA delivery to different CNS cells, such as neurons, astrocytes, and oligodendrocytes, via transvascular administration (Alvarez-Erviti et al., 2011).

The capability of RVG-9R to deliver an oligonucleotide to AchR-expressing neurons was further demonstrated by assessing the transfection efficiency of a RVG-9R-pDNA complex into an AchR-positive neuronal cell line (Neuro2a) and an AchR-negative cell line (HeLa) (Gong et al., 2012). RVG-9R delivered the pDNA (pGL3) into Neuro2a cells but not into HeLa cells; surprisingly, this delivery was more efficient than common transfection reagents. To prove the use and effectiveness of the system *in vivo*, a pGL3/RVG-9R complex (containing 50 micrograms DNA) was administered into Kuming mice via the tail vein and luciferase (Luc) expression in multiple tissues was assayed 24, 48, 72, or 120 h after injection Luc. The highest gene expression was observed in the brain after 72 h, 3 times as high as the control group (naked pGL3 plasmid), and Luc expression did not significantly change in the liver, heart, kidneys, or muscles, Luc suggesting that the brain was the primary target of the pDNA/RVG-9R complex and that the complex was internalized, despite being peripherally administered (Gong et al., 2012).

RVG29 has been more widely studied as a brain-targeting ligand for nanocarrier functionalization than as a simple complex. Nanometric cationic liposomes containing siRNA were functionalized with RVG9R to obtain siRNA-liposome-peptide complexes (LSPCs), prepared by incubating cationic liposomes first with siRNA and then with the RVG-9R peptide. The liposomes provided oligonucleotide protection from serum degradation and RVG-9R permitted the complex to selectively reach the CNS. Moreover, the complex's ability to treat prion pathology, using LSPCs carrying prion protein (PrP) siRNA, was assessed and confirmed by *in vitro* and *in vivo* studies: decreased prion infection in N2a and NCerP cell models was observed after LSPC treatment. *In vivo* studies demonstrated brain gene silencing after i.v. injection in FVB mice. Cellular prion protein (PrP^c) expression in brain cells from LSPC-treated mice was reduced to approximately 75% of control (Pulford et al., 2010).

Bare or chitosan-functionalized pluronic-based nanoparticles were conjugated with RVG29 in order to obtain nanocarriers (NC) named RVG-Bare-NC and RVG-Chito-NC, respectively, and used to study the transport of a protein drug model into the CNS (Kim et al., 2013). After intravenous administration to nude mice (mice with an inhibited immune system), both formulations reached the brain and maintained drug bioactivity; however, CNS targeting efficacy was significantly improved due to the synergistic effect of RVG peptide and chitosan and prolonged accumulation of RVG-Chito-NC was obtained. RVG-Chito-NC was selectively and highly concentrated in the brain, compared to RVG-Bare-NC, suggesting that chitosan promotes a longer circulation time by reducing macrophage uptake. The authors propose RVG-Bare-NC and RVG-Chito-NC for use asdiagnostic and therapeutic tools in brain disorders and as vehicles for the delivery of protein drugs, genetic materials, or other therapeutic agents (Kim et al., 2013).

In 2009, Liu and co-workers developed polyamidoamine dendrimers (PMAM) as carriers for genetic material to treat brain disorders by functionalizing PMAM through a bi-functional PEG spacer with the brain-targeting RVG29, obtaining PAMAM-PEG-RVG29; PAMAM-PEG-RVG29 was complexed with DNA, yielding PAMAM-PEG-RVG29/DNA nanoparticles (Liu et al., 2009). The authors observed, by *in vivo* imaging, that these nanoparticles accumulated in the brain; PAMAM-PEG-RVG29/DNA nanoparticles had specific interactions with brain capillary endothelial cells (BCEC) due to interactions with both $GABA_B$ and α7 Ach receptors. After internalization in BCEC, PAMAM-PEG-RVG29/DNA nanoparticles increased brain gene expression compared to PAMAM/DNA nanoparticles. However, an equal accumulation of PAMAM-PEG-RVG29 and PAMAM/DNA nanoparticles were observed in the spleen, suggesting that the nature of the carrier influences its peripheral distribution (Liu et al., 2009).

Hwang et al. (2011) used RVG29 to develop modified nanoparticles to carry neurogenic microRNA (miR-124a). They first showed that disulfide linkage-modified polyethylenimine (SSPEI) polymer has improved polymer biocompatibility and resists degradation by endogenous enzymes, such as glutathione reductase. Bioreducible polymers are ideal carriers for gene delivery because their disulfide bridges can be cleaved, in the cytosol, by glutathione reductase, leading to the release of the oligonucleotide or genetic material. Thus, by functionalization with RVG peptide, they used SSPEI as a carrier for miRNA transport to the brain. As a result, RVG-SSPEI nanocarriers, compared with RVG-free nanocarriers, led to an increased brain concentration of miR-124a, which was further improved when the RVG-SSPEI nanocarriers were co-administered with a mannitol solution (Hwang et al., 2011). The role of the peptide as a site-specific moiety was corroborated by another study (Son et al., 2011), where the same authors developed bioreducible PEG-polyethylenimine (PEG-PEI) nanoparticles covalently linked to RVG29 to deliver genetic material to the brain. RVG-associated PEG-PEI NPs allowed selective pDNA delivery into mouse brain after tail vein injection. The receptor-mediated uptake of pDNA into Neuro2a cells was due to the presence of nAChRs, explaining RVG-PEG-PEI passage through the BBB.

Recently, Gao et al. (2014) investigated the role of RVG29 as a brain-targeting moiety for siRNA delivery. The peptide was covalently linked to siRNA/trimethylated chitosan (TMC) complexes through bi-functional PEG. After intravenous administration in mice, the RVG-siRNA-TMC complex accumulated in the brain, demonstrating the ability to bypass the BBB and the complex's site specificity.

Itraconazole-loaded albumin nanoparticles (ITZ-NPs) were functionalized with RVG29 to study a potential treatment for intracranial fungal infection. RVG29 promoted the NPs intracellular delivery of ITZ (*in vitro* uptake studies) and increased the ITZ concentration in the brain, unlike ITZ-NPs without RVG (Chen et al., 2011).

RDP is another rabies virus glycoprotein with a different amino acid sequence than RVG-9R (YTIWMPENPRPGTPCDIFTNSRGKR ASNGGGGRRRRRRRRR) that was also fused with a 9-arginine peptide, generating RDP-9R (KSVRTWNEIIPSKGCLRVGGRCHPHVNG GGRRRRRRRRR). RDP was used to carry proteins with different isoelectric points and molecular weights (β-galactosidase (β-Gal), Luc, and brain-derived neurotrophic factor [BDNF]), to the CNS by a non-invasive approach (Fu et al., 2012a). After intravenous injection in mice, RDP enabled β-Gal, Luc, and BDNF to reach the CNS. Moreover, when tested in stroke animal models, the neuroprotective properties of BDNF were not altered when BDNF was fused with RDP. Nevertheless, RDP conjugated to BDNF has a short half-life (1 h), indicating that the system requires protection (Fu et al., 2012a). For this reason, RDP was later used to bind and deliver plasmids across the BBB. Plasmids containing the lacZ reporter (pVAX-LacZ) and BDNF (pVAX-BDNF) genes were conjugated to RDP, yielding RDP/pVAX-LacZ and RDP/pVAX-BDNF complexes, respectively (Fu et al., 2013). After confirming that the peptide could form complexes with the plasmids and that these complexes were stable in serum, RDP/pVAX-Lac Z and RDP/pVAX-BDNF complexes were intravenously injected into mice. Both complexes were not degraded in mouse serum for up to eight hours. After *in vivo* administration, RDP/pVAX-LacZ entered the neuronal cells as a RDP/DNA complex and regulated the expression of β-Gal in the brain and not in other tissues (kidney, liver, lung, heart, and spleen). No peripheral (liver and kidney) BDNF gene expression was observed after RDP/pVAX-BDNF injection; on the contrary, BDNF expression increased in the brain. RDP/pVAX-BDNF was also effective in improving some Parkinson's disease symptoms (rotation behavior) in animal models of Parkinson's disease without any evident toxicity, overall demonstrating that RDP worked as a peripherally-administered brain-targeting carrier of pDNA without modified activity (Fu et al., 2013).

Overall, peptides derived from the rabies virus, RVG and RDP, are useful tools for CNS-specific drug delivery because they are selective, non-toxic, and do not affect the pharmacological actions of oligonucleotides, genetic material, and proteins.

3. Shuttles from bacteria

The CNS is an ideal target for bacterial toxins because the toxins recognize specific CNS structures; the neurotoxins act specifically on neurons.

3.1. Cholera toxins

3.1.1. Cholera B fragment of cholera toxin (CTB)

Cholera toxin (CT) is an enterotoxin produced by *Vibrio cholerae* bacteria that is responsible for the onset of cholera, an acute diarrheal infection that is often accompanied by vomiting. Diarrhea and vomiting are due to an electrolytic imbalance associated with chloride ion efflux and reduced sodium ion influx leading to a higher water efflux from intestinal cells. CT is comprised subunits A (two domains A1 and A2, enzymatic activity, 240 amino acids) and B (5 binding subunits, 103 amino acids). The A1 domain is responsible for the cellular metabolic alterations initiated by the ADP-ribosylation of G-protein, which leads to increased levels of cyclic AMP. The non-toxic B subunit contains the binding site for ganglioside GM1 receptors, expressed in the plasma membrane of epithelial intestinal cells and throughout the CNS (Vajn et al., 2013). Because of its GM1 receptor affinity, the B subunit of cholera toxin (CTB) is currently used as a retrograde neuronal tracer. Drug delivery systems that use CTB to improve delivery to the CNS are called conjugates; all conjugate systems facilitate drug transport. Particularly, conjugation of CTB with saporin (CTB-sap), a ribosome-inactivating protein, creates complexes that selectively target myelin-producing cells in CNS that express GM1 ganglioside; CTB-sap effectively reached the CNS and induced demyelination (Ohara et al., 2005). After oral administration of a CTB-myelin basic protein complex in a triple-transgenic Alzheimer's disease mouse model, to the complex crossed the BBB and retinal barrier by passing through mucous membranes; CTB did not interfere with the therapeutic effect of myelin basic protein (Kohli et al., 2014). To improve the brain delivery of nerve growth factor (NGF) by nasal administration, a NGF-CTB conjugate was used. Because CTB can bind GM1 receptors on olfactory nerves and epithelium, it was expected to promote NGF transport toward the olfactory bulb by retrograde axoplasmic transport. In *in vivo* Parkinson's models, the conjugate improved NGF curative properties thanks to CTB enhancement of NGF transport into the brain via the olfactory nerve (Zhang et al., 2008).

3.1.2. V. cholerae DeltaG (DeltaG) fragment

DeltaG (12 kDa) is the biologically active fragment of zonula occludens toxin (Zot) protein (45 kDa), which is produced by *V. cholerae*. In the small intestine and the brain, DeltaG temporarily opens the tight junctions of cells and thus promotes the transit of substances, acting as an absorption enhancer. DeltaG was used to improve the oral bioavailability of drugs with low levels of absorption, high first-pass metabolism, and susceptibility to P-gp efflux transporters (cyclosporine, ritonavir, saquinavir, and acyclovir) (Salama et al., 2005).

Menon et al. (2005) used the DeltaG fragment to improve drug transport into the CNS. In an *in vivo* experiment, hydrophilic (methotrexate) or lipophilic (paclitaxel) model drugs with low brain distribution were co-administered with DeltaG fragments, resulting in improved drug distribution in the brain. The peptide increased passage of the drug to the brain, but the simultaneous presence of a protease inhibitor (Menon et al., 2005) and intracarotid administration were necessary to facilitate drug delivery. It would be interesting to assess the capacity of DeltaG to promote drug passage through physiological membranes after suitable functionalization of colloidal systems.

3.2. Diphtheria toxin

3.2.1. Cross-reacting material 197

Cross-reacting material 197 (CRM197) is a non-toxic mutant protein obtained from *Corynebacterium diphtheriae* diphtheria toxin which is internalized by caveolae-mediated transcytosis after binding to its receptor (diphtheria toxin receptor, DTR) (Wang et al., 2010). CRM197 has long been used in vaccines, confirming its safety in humans (Rennels et al., 1998).

Diphtheria toxin binds to heparin-binding epidermal-like growth factor (HB-EGF), which is highly expressed in some CNS diseases, such as gliomas, stroke, and seizures, but is also constitutively expressed in the BBB, neurons, and glial cells. The DTR can internalize large molecules or cargo, like proteins and liposomes,

respectively. Because of these characteristics, Gaillard et al. (2005) proposed HB-EGF as a carrier directed against DTR for specific and safe drug targeting to the brain. After fusion with a model protein of 40 kDa (horseradish peroxidase), CRM197 promoted receptor-mediated endocytosis through the BBB and allowed specific targeting of the brain both in *in vitro* BBB models and after intravascular injection in guinea pigs without toxicity. Recently, nanoparticles were functionalized with CRM197 to deliver drugs to the brain.

Polymeric nanoparticles, based on polybutylcyanoacrylate (PBCA) and functionalized with CRM197, crossed BBB models *in vitro* and carried zidovudine through the barrier (Kuo and Chung, 2012). PEG-PEI/siRNA nanoparticles were also functionalized with CRM197 and designed for glioblastoma therapy, using siRNA targeting the growth factor pleiotrophin (PTN-siRNA) as a therapeutic agent. After intraperitoneal injection in glioblastoma model mice, the glioma-directed formulation, CRM197-PEG-PEI/siRNA, was therapeutically effective, while the CRM197-lacking control did not (Höbel et al., 2011).

However, in the studies mentioned above, even if CRM19 is a good CNS carrier moiety, they did not investigate its distribution in peripheral tissue; the DTR is also expressed in the peripheral nervous system (Nakamura et al., 2001).

3.3. Clostridial toxins

Clostridium botulinum and *Clostridium tetani* produce very potent toxins, the tetanus toxin (TeNT) and botulinum toxin (BoNT), respectively. These toxins are metalloproteases that act on nerve terminals and inhibit exocytosis (Popoff et al., 2010). BoNT targets motoneuron endings while TeNT initially targets peripheral nerves and thereafter reaches the CNS, migrating in a retrograde pathway along the axons.

The specific actions against nerve cells justify the use of clostridial toxins as potential CNS delivery systems. The clinical use of tetanus toxin as a drug delivery system is limited because the majority of the population is vaccinated against *C. tetani* (Singh et al., 2010). Nevertheless, the heavy chain of TeNT was used as a delivery vehicle for drugs and ligands to brain neurons (Bizzini et al., 1980; Francis et al., 1995).

BoNT was generally assumed to interact almost exclusively with the peripheral nervous system, even if there was evidence that botulinum neurotoxin type A (BoNT/A) can directly affect the CNS, as recently reviewed by Mazzocchio and Caleo (2015); however, the mechanism remains to be elucidated. The most accepted theories predict central plastic rearrangement or alteration of sensory input; direct crossing of the BBB is unlikely, due to the high molecular size of the compound (Caleo and Schiavo, 2009).

3.3.1. Botulinum neurotoxin (C. botulinum)

BoNTs target the peripheral neuromuscular junctions (NMJ), where they are capable of blocking acetylcholine release, leading to the most obvious symptom of botulinum intoxication: flaccid paralysis. 7 serotypes of BoNTs (A–G) have been identified, with comparable structures and mechanisms of action (Li and Singh, 1999). BoNT is a protein in which it is possible to distinguish a disulfide bridge interaction between a light chain (LC, 50 kDa) and a heavy chain (HC, 100 kDa). LCs and HCs act differently: the LC, with its metalloprotease domain, is responsible for the toxicity of the neurotoxin, while the HC promotes specific and selective binding of the neurotoxin at nerve endings and subsequent translocation of the LC (Singh, 2000; Li and Singh, 1999). Because the HC of BoNT delivers the toxic LC, removing the LC from BoNT allows use of the HC to transport pharmacologically-active molecules to the CNS.

The HC of BoNT possesses several characteristics that make it attractive as a drug delivery vehicle: high neuronal specificity and high receptor binding affinity with subsequent internalization in nerve terminals. The BoNT HC contains a translocation domain that can allow the transport of drugs and macromolecules into the cytosol while escaping endosomal vesicles. Furthermore, since each identified serotype binds to a different pool of receptors, the several identified BoNTs offer the opportunity to access various neuron-specific markers.

Chimeric non-toxic BoNTs were used as tools for targeted protein delivery (Bade et al., 2004; Band et al., 2010; Vazquez-Cintron et al., 2014; Zhang et al., 2009). The BoNT HC was used to treat botulism poisoning by Zhang et al. (2009). A 10-kDa dextran, chosen as a model therapeutic molecule of toxin inhibitors, was linked to a non-toxic recombinant HC of BoNT-A (rHC) (Zhang et al., 2009) to obtain a drug delivery vehicle (DDV) that could efficiently deliver a drug specifically to presynaptic nerve terminals for treating botulinum poisoning. In this DDV, the BoNT-A was used to selectively target BoNT-sensitive cells and promote DDV internalization; this DDV was studied in spinal cord primary cells in culture to elucidate the internalization mechanism. Results showed that the DDV was internalized by endocytosis due to the interaction of rHC with the BoNT-A receptor. The rHC fragment remained in the endosome vesicles and the drug component was released into the cytosol in a neuronal maturation-dependent way; the drug carrier separation was of 20 ± 3%, 32 ± 5%, and 40 ± 5% in the 1-, 2-, and 3-week cell growth periods, respectively. Because rHC was in the endosomes, it is not responsible for BoNT-A poisoning, since BoNT-A acts in the cytosol. On the other hand, the drug does not remain entrapped in exosomes but is released into the cytosol, meaning that it is free to exert its action. This DDV was designed for botulinum poisoning treatment but was also proposed as a strategy to develop new site-specific neuro-therapeutic tools (Zhang et al., 2009).

Full-length botulinum neurotoxin type D (BoNT-D) (Bade et al., 2004), contains binding and translocation domains but is proteolytically inactivated, was proposed to deliver functional proteins to the neuronal cytosol by attaching the cargo proteins to the amino terminus of BoNT-D (Bade et al., 2004). Band et al. (2010), obtained, using BoNT-A, several non-toxic compounds that kept the same mechanism of action as the original fragment. The obtained compounds were proposed as carriers of therapeutic drugs to the neuronal cytoplasm.

In a very recent work, the same authors studied the internalization and intracellular trafficking properties of BoNT-A *ad,* a non-toxic derivative of wild-type BoNT-A, in primary hippocampal neuron cultures. BoNT-A *ad* selectively targeted primary hippocampal neuron cultures rather than glial cells (Vazquez-Cintron et al., 2014). Its non-toxic LC can reach cellular cytoplasm via the trafficking mechanism of native BoNT, suggesting its potential use as a system to address multiple medical and biodefense needs.

BoNT, in the form of the products Botox® (2000) and Myobloc™ (2000), has been approved by FDA, making the path for approval of new products easier.

3.3.2. Tetanus neurotoxin (C. tetani)

In 1980, the tetanus toxin (TeNT) was proposed, for the first time, as a drug carrier for the treatment of CNS diseases (Bizzini et al., 1980). TeNT binds to different peripheral nervous system cell types (sensory, motor, and autonomic neurons) and CNS fibers and reaches the CNS by retrograde transport.

A non-toxic C-fragment of TeNT (TTC), obtained from the C-terminus moiety of the TeNT HC, reaches the NMJ and penetrates the motoneurons, from which it is able to move by retrograde axonal transport; thus, TTC could facilitate the transport of desired

therapeutic molecules to the CNS (Toivonen et al., 2010; Price et al., 1975).

For the potential treatment of lysosomal storage CNS diseases, in which enzyme deficiencies are observed, a TTC fragment, B-IIb, was conjugated to a model enzyme (glucose oxidase) and studied in terms of the ability to reach spinal-cord motoneurons through retrograde transport from axon terminals. When the conjugate was intramuscularly injected in mice, the conjugate did reach spinal cord motoneurons without any alteration in enzymatic actions, demonstrating that B-IIb could serve as a vector for enzyme transport into the CNS after peripheral administration (Beaude et al., 1990).

In order to improve delivery, bioavailability, and parenchymal concentration of proteins into CNS neurons, TTC was associated with a human Cu/Zn superoxide dismutase and administrated intracerebroventricularly (Benn et al., 2005); this association increased the protein's availability in the CNS not by overcoming the BBB, but allowing the targeting of neurons in the brain parenchyma through the cerebrospinal fluid. The authors suggested this invasive administration route, instead of peripheral administration because TTC has a long lifetime in mouse brain after intraparenchymal injection and problems due to immunization of a large part of the population against TeNT should be avoided. Anti-TTC antibodies (due to vaccination) can neutralize TTC prior to its binding of the NMJ. Further, although TTC interacts with nerve-cell endings after peripheral administration, it is not able to reach CNS in high concentrations because not all neurons have CNS projections outside the BBB (Benn et al., 2005).

The C fragment was designed as a drug carrier to exploit how TTC moves via axonal transport from motoneurons of the NMJ (Bizzini et al., 1977) to the motorneurons of the spinal cord and brainstem; this system was designed to treat neuromuscular diseases, such as ASL or Parkinson's. The C fragment was conjugated with a glial cell line-derived neurotrophic factor (GDNF) and intramuscularly injected into mice; drug accumulation in spinal motoneurons and consequent neuroprotection occurred (Larsen et al., 2006).

Motoneuron survival was observed *in vitro* after TTC conjugation to cardiotrophin-1, suggesting effective delivery. TTC, thanks to its site-specificity, confers targeted delivery of cardiotrophin-1 with a selective uptake by motoneurons and cerebral neurons and no interactions with glia, hepatocytes, or cardiomyocytes, reducing side effects like weight loss and cardiac hypertrophy (Bordet et al., 2001).

Other studies involved TTC fusion protein with B-cell lymphoma extra-large protein (Bcl-xcL) (Carlton et al., 2008), insulin-like growth factor 1 (IGF1) (Payne et al., 2006), or BDNF (Roux et al., 2006). In all cases, specific internalization and retrograde neuron transport occurred with retention of the biological activity of the peptides.

Taking into account these remarks, the TTC fragment might be a useful vector for several CNS diseases. Unfortunately, its application in human clinical practice is still limited because a high percentage of the population is vaccinated against TeNT (Toivonen et al., 2010).

3.3.3. Epsilon toxin (*Clostridium perfringens*)

C. perfringens is a rod-shaped, anaerobic, Gram-positive, and spore-forming bacterium. *C. perfringens* is sub-categorized into five different strains (A–E), producing a unique spectrum of toxins; strains B and D synthesize alpha and beta toxins and alpha toxins, respectively. However, in both cases, the epsilon toxin (ETX) is the major virulence factor. *C. perfringens* is a normal inhabitant of human and animal intestines but *C. perfringens* intoxication can occur after ingestion of food contaminated with high amounts of bacteria, which produce active toxins inside the intestine (http://www.cdc.gov/foodsafety/clostridium-perfingens.html).

High quantities of these saccharolytic intestinal bacteria are in ruminants after ingestion of large amounts of starch; thus, the production of an inactive prototoxin (formed by 311 amino acid) increases. This prototoxin becomes ETX by proteolytic cleavage of a small peptide (14 amino acids) from the N-terminus (http://www.cfsph.iastate.edu/Factsheets/pdfs/epsilon_toxin_clostridium.pdf; Zhu et al., 2001). The toxin, through opening the intestinal tight junctions, passes into the circulatory system and reaches different organs, including the brain. After crossing the BBB, ETX stimulates glutamate release, responsible for the CNS excitotoxicity observed in animal enterotoxemia, which includes hyperesthesia, convulsions, paddling, opisthotonus, loss of consciousness, cerebral edema, and dyspnea. ETX is not strictly a neurotoxin, because it acts on different cell types, but it is able to bypass the BBB and accumulates in the brain with high affinity (nM range) (Nagahama and Sakurai, 1992). Passage through the BBB is attributable to an interaction with an unknown receptor present both in the brain vasculature and myelinated brain regions. This interaction allows ETX to integrate into the plasma membrane as a heptameric pore. ETX accumulation in the brain can increase vascular permeability at the level of the BBB (Bokori-Brown et al., 2011; Rumah et al., 2013).

For this reason, neuronal damage can occur, characterized by progressive cytoplasmic vacuolization plus necrosis (Popoff et al., 2010).

Administration of the ETX precursor (ETXp) in a rat model reduces the endothelium barrier antigen in rat brain endothelial cells, accompanied by reversible opening of the BBB (Zhu et al., 2001). ETXp was used at a non-toxic dose and injected intraperitoneally to deliver bleomycin to the brain for the treatment of gliomas. Photochemical internalization (PCI) was used to potentiate BBB opening by ETXp only in the site exposed to the light, preventing extensive actions of the prototoxin on the BBB. Despite that, the method enabled a temporary and localized opening of the BBB and, consequently, bleomycin effectively reached the site of action; nevertheless, its application in human therapy is limited because of the toxicity of ETXp (Hirschberg et al., 2009). ETXp toxicity is due to greater conversion of the precursor to the corresponding toxin, caused by uptake of macrophages that promote the proteolytic cleavage at the lysosomal level. In addition, very recently the toxin has been considered responsible for the onset of multiple sclerosis that is characterized by BBB permeability and demyelination (Rumah et al., 2013), making its application more problematic.

4. Shuttles from animals

4.1. Candoxin (CDX)

Candoxin is a three-finger toxin from the Malayan krait *Bungarus candidus* snake. It consists of a single polypeptide chain of 66 amino acids with 5 disulfide bridges (Nirthanan et al., 2002, 2003). Candoxin produces a poorly-reversible block of α7 nAChRs in electrophysiological experiments. Nirthanan et al. (2002, 2003) suggest its use as a biological marker of α7 nAChRs.

Paclitaxel-loaded poly(ethylene glycol)-poly(lactic acid) (PEG-PLA) co-polymer micelles were functionalized with a 16-residue peptide derived from the loop II region of candoxin (Zhan et al., 2011). The peptide binds the α7 nAChR, richly expressed in the endothelial cells of the BBB. The micelles (39 nm) showed a hydrophobic core, loaded with the active compound, and a hydrophilic shell. The drug delivery system was functionalized with CDX and tested for the treatment of glioblastoma multiforme (GBM), one of the most frequent primary malignant brain tumors.

Micelles shuttled by CDX reached the brain; micelles without a functionalized delivery system did not and paclitaxel's activity was retained. In another study, paclitaxel micelles functionalized with CDX were co-administered with TRAIL nanoparticles (tumor necrosis factor-related apoptosis-inducing ligand) to enhance their anti-tumor efficacy (Zhan et al., 2012). Authors demonstrated that a combination of targeted gene therapy with a non-viral gene carrier is an attractive and efficient strategy to treat GBM (Zhan et al., 2012).

4.2. Ophiophagus Hannah toxin B

Toxin B is contained in the venom of the King Cobra (*Ophiophagus hannah*) snake, the world's longest venomous snake. Toxin B is 73 amino acids long and contains 5 disulfide bridges. The 3-D structure of toxin B consists of 3 hairpin-type loops emerging from a globular head. Among the 3 loops, loops I and III are shorter than loop II (middle loop), which comprises residues 19–43. The 3 loops are tethered together by 4 disulfide bridges. Many residues located at the tip of loop II are either strictly conserved or only conservatively substituted among short and long neurotoxins. Loop II has an important role in the toxicity of the long toxins (Peng et al., 1997). It belongs to the three-finger snake neurotoxins, in which the loop II segments are responsible for the interaction with nAChRs. Because of this feature, they are considered neurotoxins; nAChRs are abundantly expressed in the CNS and the BBB.

KC2S is a synthetic peptide derived from toxin B that binds to nAChRs with high selectivity. nAChR selectivity was also observed when KC2S was coupled with polymers to form paclitaxel-encapsulating KC2S-PEG-PLA micelles. In fact, these systems, due to the specific interaction of KC2S with nAChRs, demonstrated brain-targeting capability after intravenous administration (Zhan et al., 2010).

4.3. Polylysine-molossin

Polylysine-molossin is a 31-amino acid synthetic peptide derived from the molossin venom of the snake, *Crotalus molossus molossus*, and consists of two main domains: a sequence of the 15-amino acid integrin-targeting domain at the carboxy terminus and a 16-lysine chain for electrostatic binding of DNA at the amino terminus (Collins et al., 2000, 2003).

Collins et al. (2000) demonstrated that this peptide can strongly bind all parts of the CNS of rats and, in 2003, they used the peptide as a vector for gene delivery to primary neuron cultures. However, to study the transfection efficiency in the primary neuronal cell line, the carrier was used in combination with an endocytic escape agent (chloroquine), a synthetic fusogenic peptide from influenza virus (haemoagglutinin)_or a lipid transfection agent (Lipofectamine 2000). The transfection efficiency of polylysine-molossin/DNA complexes alone was almost zero. Besides increasing the transfection efficiency, the peptide can target not only nerve cells and the CNS, but also other cells and tissues (Collins et al., 2000). Thus, peripheral administration *in vivo* could determine if it targets non-CNS structures, thereby negating its use as a vector for CNS-specific targeting.

4.4. Chlorotoxin

Native chlorotoxin (ClTX) was originally identified in the venom of the scorpion, *Leiurus quinquestriatus hebraeusis,* and currently it is available in a recombinant form from *E. coli.* ClTX is a 4 kDa, 36-amino acid neurotoxic peptide; the peptide binds a lipid raft-anchored complex that contains matrix metalloproteinase-2 (MMP-2), membrane type-I MMP, transmembrane inhibitor of metalloproteinase-2 (TIMP2), ClC-3 chloride ion channels, and other proteins (Veiseh et al., 2007; Deshane et al., 2003). ClTX is very toxic for invertebrates but not mammals, and ClTX can cross the BBB. ClTX binds MMP-2, which is specifically up-regulated in gliomas and related cancers (DeBin et al., 1991). ClTX was first studied as a diagnostic marker for glioma cells but was simultaneously proposed for the treatment of glioblastoma as a site-specific delivery system. ClTX can selectively recognize brain tumors (gliomas) rather than normal cells (Soroceanu et al., 1998; Lyons et al., 2002), which led to thorough investigation of ClTX for the treatment and diagnosis of several cancers.

ClTX's small dimensions make it ideal for intracranial delivery and its tightly-folded structure increases BBB permeability.

A review written by Fu et al. (2012b) on ClTX-conjugated nanoparticles as potential glioma-targeted drugs, discussed the advantages of the peptide in the effective targeted therapy of glioma. Copolymer coated-ClTX-Cy5.5 and nanoparticle-DNA-ClTX are some reviewed examples of delivery systems able to selectively target brain tumors *in vivo;* PEI-siRNA-ClTX nanoparticles show similar behavior but in *in vitro* experiments.

Doxorubicin-loaded liposomes (100 nm) functionalized with ClTX were proposed for glioblastoma treatment or diagnosis. ClTX highly facilitated the uptake of liposomes by 3 glioma cell lines and 1 endothelial cell line, demonstrating that the presence of ClTX increases doxorubicin cytotoxicity against glioma cells (Xiang et al., 2011). *In vivo* studies confirmed that ClTX-modified liposomes accumulate in subcutaneous and intracranial tumors; the same liposomal formulations were afterwards proposed as a drugdelivery system into the brain to improve Parkinson's disease therapy. In fact, liposomes highly facilitated levodopa uptake by brain microvascular endothelial cells *in vitro* (Xiang et al., 2012).

Costa et al. (2013) developed liposomes for targeted delivery of nucleic acids to glioblastoma by covalently coupled ClTX liposomes encapsulating antisense oligonucleotides (asOs) or small interfering RNAs (siRNAs). After intravenous administration, liposomes (180 nm) covalently coupled with ClTX reached intracranial tumors (Costa et al., 2013), confirming the central role of ClTX for specific targeting to glioblastoma. Nanoprobes comprised of ClTX reached brain tumors in a genetically engineered mouse model (Veiseh et al., 2009).

TM-601, a synthetic version of ClTX, started Phase I clinical trials in 2006; the goal was to determine if 131-I-labeled TM-601 (chlorotoxin) was a useful diagnostic tool in radiographic imaging to identify primary solid tumors with evidence of metastatic involvement (https://clinicaltrials.gov/show/NCT00379132).

4.5. Apamin and mellitin (Apis mellifera)

Apamin is a highly neurotoxic 18-residue polypeptide found in apitoxin, a venom of the bee (*A. mellifera*). Apamin poisoning symptoms are mainly convulsions and respiratory arrest. It strongly selectively inhibits low-conductance Ca^{2+}-activated K^+ channels (Hugues et al., 1982) expressed in several cell types, including cells in the CNS, intestinal myocytes, endothelial cells, and hepatocytes.

Oller-Salvia et al. (2013) recently demonstrated the ability of apamin and its non-toxic analogue (ApOO) to pass through an *in vitro* BBB model. The authors excluded passage of substances by simple diffusion (PAMPA test) as the mechanism of action and suggest active transport with exclusion of transcytosis by adsorption, because the 2 peptides are weakly charged at physiological pH. They also hypothesized a specific cell passage, as the peptides did not permeate through a monolayer Caco-2 cell model. Due to the resistance of the 2 peptides to the various pHs, temperatures, serum proteases, and coefficients of permeation, similar to other shuttle peptides, apamin and ApOO were proposed as potential carriers for drug delivery to the CNS (Oller-Salvia et al., 2013).

However, they do not specify the nature of the cargo that they would be able to convey nor any specific passage mechanism. Currently, despite its potential, there is no literature about the use of ApOO in delivery systems as shuttles due to its recent investigation in the CNS field studies.

Forty-fifty percent of the dried venom of *Apis millifera* is represented by mellitin, a 26-amino acid hemolytic peptide that can form pores in the membrane lipid layer, increasing its permeability (Gomara et al., 2003).

For gene delivery to the brain, polyplexes that contained incubating plasmid DNA and mellitin-grafted diblock copolymers (pHgMel*b*HK10) were proposed (Schellinger et al., 2013). pHgMel*b*HK10 consisted of N-(2-hydroxypropyl) methacrylamide block – HPMA – for mellitin conjugation and pyridyl disulfide methacrylamide block – PDSMA – for DNA binding. In *in vitro* gene delivery studies, polyplexes with a polymer/DNA charge ratio (N/P) of 3, comprising 5–15% of pHgMel*b*HK10 and delivering the Luc reporter gene, showed ~2 orders of magnitude higher Luc activity compared to non-mellitin grafted control pHK10 polyplexes (Schellinger et al., 2013). In PC12 cells, the highest transfection efficiency was 2 orders of magnitude higher than pHK10 and achieved by a mixed formulation containing 15% pHgMel*b*HK10, with no apparent cytotoxicity. The authors indicated that defined N/P, as mellitin concentration increased in polyplexes, did not lead to greater transfection efficiency but enhanced cytotoxicity. pHK10/ pHgMel*b*HK10 15% and pHgMel*b*HK10 copolymers formulated at N/P = 3 were injected into the right lateral ventricles of mice to deliver the Luc plasmid. There were statistically significant increases ($p < 0.02$) in Luc activity for both pHK10/ pHgMel*b*HK10 15% (5-fold higher) and pHgMel*b*HK10 polyplexes (35-fold higher), compared to pHK10. Although the system was proposed for gene delivery to the brain, it was not selective for neuronal cells, as it did not discriminate between HeLa or PC12 cells; it was also cytotoxic, related to mellitin content. Brain-specific targeting occurs only after an invasive intraventricular administration. The authors propose the polyplexes for gene delivery to the brain, but *in vivo* studies on toxicity and the specificity of brain targeting are not thoroughly investigated. As originally pointed out by the authors, the use of mellitin was based on allowing endosomal escape for gene delivery but not for specific targeting (Schellinger et al., 2013).

5. Prospects for the future

Although there are many neurotoxins in nature, research on their use in the CNS delivery systems is still limited; for example, there are no mentions of the use of neurotoxins from plants despite the many plants that produce neurotoxins. Xxamples of neurotoxic plants include *Mandragora officinalis*, *Datura stramonium*, *Conium maculatum*, *Coriaria myrtifolia*, *Ricinus communis, Podophyllum pelatum, Blighia sapida*, *Cycas circinalis,* and *Lathyrus sativus*. Fungal neurotoxins, such as fumonisin B_1 (*Fusarium moniliforme*), slaframine and swainsonine (*Rhizoctonia leguminicola*), or lolitrems (*Acremonium lolii*), are also unmentioned.

The vast world of neurotoxins and their uses as shuttles in delivery systems is still to be explored. Venoms and neurotoxins are often studied for therapeutic purposes rather than as drug delivery systems; scientific research on drug targeting to the brain is not yet very active. There is still a very long way before reaching satisfactory results. The increased incidences of CNS disorders suggests that effort should be increased to further study known neurotoxins and identify new non-toxic fragments with CNS specificity. Researches carried out so far with neurotoxins of microbial and animal origin have given encouraging results, in both *in vitro* and *in vivo* studies, for specific targeting to the CNS.

Scientific research can take advantage of the mechanisms already present in nature to overcome some major obstacles, such as the BBB.

Conflict of interest

The authors declare no conflict of interest.

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