Trends in Molecular Medicine

CellPress

Review

Circumventing Brain Barriers: Nanovehicles for Retroaxonal Therapeutic Delivery

Saak V. Ovsepian,[1,2,3,*] Valerie B. O'Leary,[4]
Vasilis Ntziachristos,[1,2] and J. Oliver Dolly[3]

In addition to safeguarding the central nervous system (CNS) from the vast majority of pathogens and toxins, transvascular barriers impose immense challenges to the delivery of beneficial cargo. A few toxins and neurotropic viruses capable of penetrating the brain have proved to be potentially valuable for neuron targeting and enhanced transfer of restorative medicine and therapeutic genes. Here we review molecular concepts and implications of the highly neurotropic tetanus toxin (TeTx) and botulinum neurotoxins (BoNTs) and their ability to infiltrate and migrate throughout neurons. We discuss recent applications of their detoxified variants as versatile nanovehicles for retroaxonal delivery of therapeutics to motor neurons and synapses. Continued advances in research on these remarkable agents in preclinical trials might facilitate their future use for medical benefit.

Trends

In addition to the maintenance of electrolyte, metabolic, and transmitter homeostasis, blood–brain barriers (BBBs) impose a major challenge to therapeutics delivery in the central nervous system (CNS).

A few pathogens and neurotoxins are nevertheless capable of crossing BBBs and thus certain neurotoxins may represent promising agents for the targeting and delivery of therapeutics to the CNS.

Clostridial neurotoxins (CNTs) – botulinum toxins and tetanus toxin – are among the most potent biological poisons, owing to their outstanding neurotropism and capacity to infiltrate neurons.

The exceptional neurotropism of CNTs and their ability to penetrate and propagate within nerve cells have been recently co-opted for targeting therapeutics to various neuronal compartments; detoxified full variants of CNTs appear to be superior to binding fragments in these processes.

Fusion proteins of innocuous CNTs with core streptavidin have been developed as versatile carriers for the transfer of biotinylated payloads and therapeutic genes to neurons and synapses.

Delivering Therapeutics to the CNS: Crossing the Defense Line

Delivery of therapeutic cargo and vectors to central neurons, while medically desirable, is also a daunting task. Despite major progress in the design and preclinical validation of novel neurotherapeutic candidates, their clinical use remains elusive. This is largely due to the lack of effective means for their transfer to the CNS. Unlike other organs, the brain and spinal cord are enveloped by transvascular barriers, which maintain stable concentrations of ions, hormones, and metabolites in addition to protecting neurons from pathogens and toxins. This specialized system of vascular sieves is highly conserved and prevents the penetration of nearly 98% of small and 100% of large molecules [1,2]. It is not surprising, therefore, that the majority of synthetic payload, vectors, and other biomaterials with therapeutic potential fail to cross the **blood–brain barrier (BBB)** (see Glossary) to reach the brain.

To enable selective exchange of nutrients and metabolites but counter pathogens and toxins in gaining entry into the CNS, the BBB has adopted specialized transport mechanisms [3,4]. Millions of years of struggle between pathogen and host have nevertheless facilitated the rise of complex mechanisms for the former to cross the defense lines of the brain. Specifically, studies have demonstrated that to gain entry, select pathogens take advantage of: (i) transcellular or paracellular transport systems of the vascular endothelium; (ii) the retroaxonal transport machinery of central neurons projecting to the periphery; or (iii) immune cells surveying the CNS [1,3,5]. While much research has been conducted on preventing pathogens and toxins from infiltrating the CNS, recently the focus has shifted towards exploring the CNS entry and neuron targeting mechanisms used by select pathogens, to exploit the possibility of achieving transfer of beneficial cargo [2,4]. In particular, retroaxonal delivery of beneficial genes has emerged as a promising approach for target-driven therapeutics and **theranostics** [6–11] (Box 1).

[1]Institute for Biological and Medical Imaging, Helmholtz Zentrum Munich, German Research Center for Environmental Health, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany

 http://dx.doi.org/10.1016/j.molmed.2016.09.004
© 2016 Elsevier Ltd. All rights reserved.

Box 1. The Clinician's Corner

- The seven serotypes of BoNTs and TeTx are known as the deadliest of all biological poisons, yet have also emerged as promising therapies for numerous medical conditions. Their wide clinical applications to subdue hyperactive secretory cells and relax tense muscles have been extended to use as nanocarriers for enhanced biotherapeutic delivery to MNs and synapses.
- Due to their special molecular characteristics, BoNTs and TeTx show preferences for specific neuron types, neuronal compartments, and functions. These features render detoxified CNTs and their fragments highly suitable for targeting different neurons and neuronal compartments with selective modulation of their functions.
- Despite the general consensus that C-terminal fragments are the sole determinant of neurotropism and the post-endocytic sorting of CNTs to various compartments, increasing evidence suggests that detoxified full-length CNTs might outperform the fragments in several processes. These findings suggest close cooperation between CNT domains, which might contribute to their full performance as powerful toxins and nanocarriers.
- Research advances in the field of chimeric CNTs have revealed several unique characteristics of various fragments including preferential targeting to different neuron types and toxicity; compelling evidence suggests the importance of cooperation between different domains in determining the potency and toxicity sites of CNTs.
- These developments have opened new prospects for harnessing the full therapeutic capabilities of CNTs for preclinical and clinical applications.
- While concerns remain over the residual toxicity of detoxified CNTs, advances in animal studies and recombinant technologies inspire optimism in the future use of CNTs as carriers for retroaxonal delivery of cargo and therapeutic genes to the brain. The latter should facilitate the validation of candidate neurotherapeutics and their application for the amelioration of neuronal or synaptic dysfunction in pathological conditions that might include chronic neurodegenerative diseases of MNs.

[2]Munich School of Bioengineering, Technical University Munich, 81675 Munich, Germany
[3]International Centre for Neurotherapeutics, Dublin City University, Dublin 9, Ireland
[4]Institute of Radiation Biology, Helmholtz Zentrum Munich, German Research Center for Environmental Health, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany

*Correspondence:
saak.ovsepian@gmail.com
(V. Ovsepian).

As the sole peripheral synapse of motor neurons (MNs), neuromuscular junctions (NMJs) represent the primary entry site for several pathogens and toxins. Due to extension beyond the BBB, MN axons and NMJs are accessible to both the lymphatic and blood circulatory systems, where presynaptic nerve terminals are enriched with specialized membrane lipids and synaptic vesicle proteins facilitating the binding and uptake of specific microbes and neurotoxins. Synaptic vesicle proteins undergo constant turnover and are related to retroaxonal transport systems, linking the NMJ to MN soma located in the CNS (Figure 1A,B). These arrangements are critical for the special vulnerability of MNs to several neurotoxins and viruses with peripheral or central effects. The physiological processes and mechanisms governing synaptic functions as well as local and long-range effects of neurotoxins and pathogens in MNs have been the subject of numerous excellent reports and reviews [12–17].

Here we review the molecular and cellular premise that the outstanding neuronal tropism of TeTx and BoNTs may render these toxins highly promising nanocarriers for the delivery of therapeutics to MNs. A brief and timely appraisal of the binding, uptake, and retroaxonal transport of these toxins is presented with considerations for targeting neurons and synaptic functions. Recent evidence is discussed on the role of dual TeTx receptors as well as on the **retrograde transport** of BoNTs and TeTx (and their subdomain interactions) in mediating effective neurotoxicity and payload transport/delivery. Finally, new reports advocating the use of full-length detoxified TeTx and BoNTs as transport **nanovehicles** are outlined, with a consideration of their possible impact on advancing therapeutic targeting and delivery to MNs.

BoNTs and TeTx: A Brief Overview

Clostridial neurotoxins (CNTs) (seven serotypes of BoNT (A–G) and a single TeTx) bear remarkable pathogenicity towards MNs, which can lead to severe neuroparalysis with high mortality at picomolar concentrations. This has not only labeled them as the deadliest of all known biological toxins but has also highlighted their enormous potential as some of the most promising neuron-targeting vehicles for therapeutic delivery to nerve cells and synapses. Produced by anaerobic ***Clostridium botulinum*** or ***Clostridium tetani***, BoNTs and TeTx are multidomain proteins [molecular weight (MW) = ~150 kDa] comprising a heavy chain (HC) containing H_C acceptor-binding and H_N translocation domains (MW = ~50 kDa each) and a

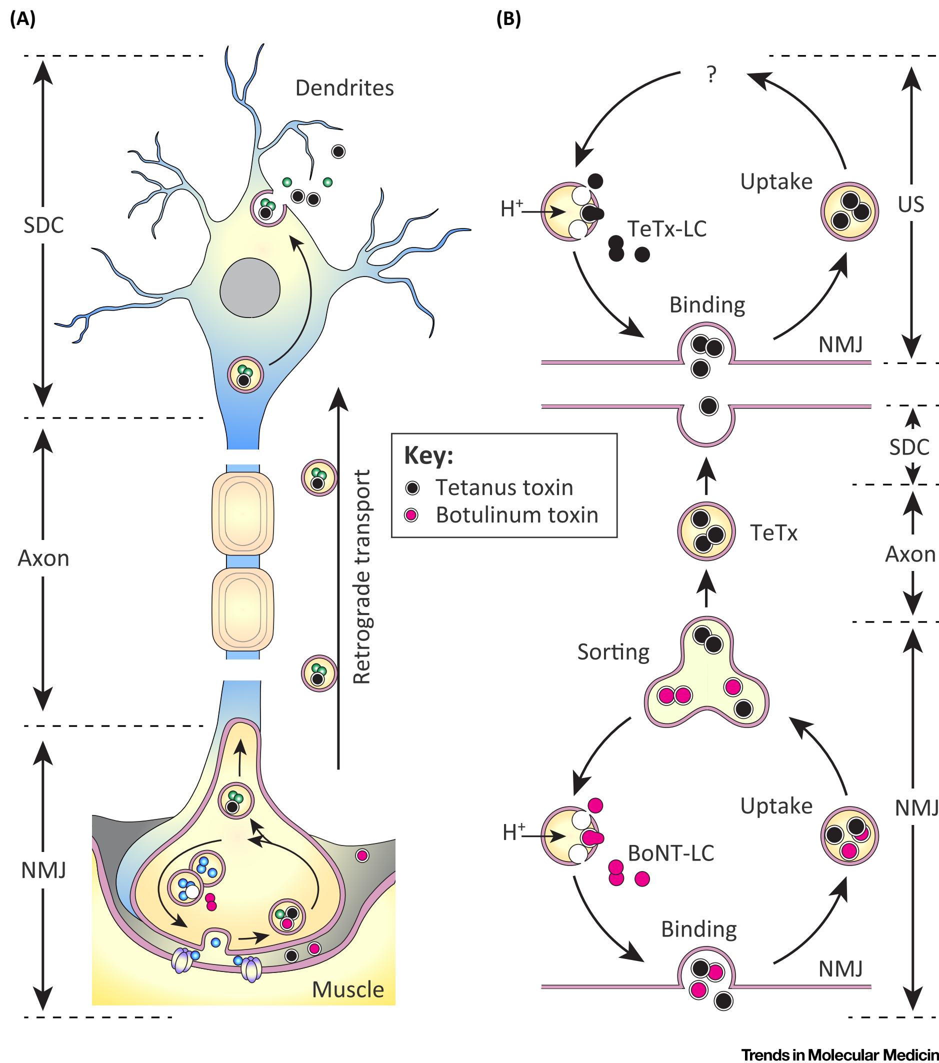


Figure 1. The Journey of Botulinum Neurotoxins (BoNTs) and Tetanus Toxin (TeTx) into Motor Neurons. (A) Schematic of a motor neuron illustrating three main compartments: the neuromuscular junction (NMJ), the axon, and the somatodendritic compartment (SDC). (B) After infiltrating motor neurons at the NMJ, BoNTs take the local synaptic vesicle journey, translocating the proteolytic light chain (LC) from acidifying vesicles and causing a synaptic block. TeTx, by contrast, is sorted to retrograde transport carriers to reach the SDC of motor neurons. Through dendritic release followed by endocytosis, TeTx enters upstream synapses (US) by crossing into inhibitory interneurons, where TeTx-LC is released and blocks synaptic transmission. Whether TeTx propagates further up from these primary interneurons remains to be shown.

light-chain (LC) protease (MW = ~50 kDa) [12,18,19] (Figure 2A). Unlike botulinum poisoning, which is related to consumption of BoNTs contaminated food, TeTx intoxication is caused by wound infections with germinating *C. tetani* spores and the release of toxic substances into bodily fluids. On entry into blood and lymph, BoNTs and TeTx target MN synaptic terminals, infiltrating them via receptor-mediated uptake [16,20,21]. This initial step is followed by translocation of the proteolytically active LC into the cytosol at the entry site (i.e., the NMJ) or upstream in primary sensory and MN synapses with cleavage of **soluble NSF-attachment protein receptors (SNAREs)**, after which synaptic failure and paralysis of the infected organism occur. Despite fundamental structural similarities and common entry sites, all BoNTs exert their deadly effects by arresting synaptic transmission at the NMJ (Box 2), whereas TeTx exploits fast axonal

Glossary

Blood–brain barrier (BBB): a system of selective permeable vascular barriers separating the circulating blood from extracellular fluids of the CNS.

Clostridial neurotoxins (CNTs): a collection of highly potent and neuron-specific toxins produced by *C. botulinum* and *C. tetani*.

***Clostridium botulinum* and *Clostridium tetani*:** Gram-positive, anaerobic spore-forming bacteria with the ability to produce the A–G serotypes of BoNTs and TeTx, respectively.

Core streptavidin (CS): shortened version of streptavidin comprising 13–139 amino acids with maintained high affinity of binding to biotin.

C-terminal fragments: H_C- and H_{CC}-binding fragments of the BoNT and TeTx HC used for neuron targeting.

Dual-acceptor model of CNT binding to nerve terminals: a model proposed by Montecucco describing the two-step binding of CNTs to nerve terminals [22]. First, CNTs associate with polysialogangliosides at the surface membrane, which leads to the accumulation of CNTs at nerve terminals. This initial enrichment on the neuronal membrane is followed by the interaction of CNTs with thinly distributed protein receptors while still bound to the gangliosides. Such simultaneous associations with gangliosides and protein receptors would be considered high-affinity CNT binding to nerve endings, facilitating their activity-dependent endocytosis within synaptic vesicles.

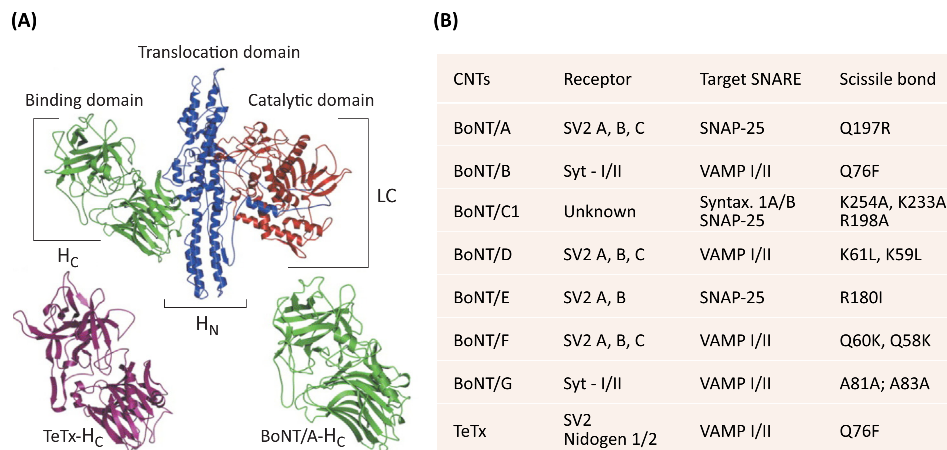
Endocytosis: a form of cellular transport in which molecules of interest bound to the surface are engulfed by a cell through an energy-dependent process.

Flaccid and spastic paralysis: abnormal conditions characterized by: (i) weakness or unresponsiveness; or (ii) involuntary contraction of a group of muscles. Both states are associated with loss of muscular function.

Ganglioside: a molecule comprising a glycosphingolipid (ceramide and oligosaccharide) with one or more sialic acids linked on the sugar chain.

Glycine- and GABA-ergic interneurons: two principal subclasses of inhibitory spinal cord

CellPress



CNTs	Receptor	Target SNARE	Scissile bond
BoNT/A	SV2 A, B, C	SNAP-25	Q197R
BoNT/B	Syt - I/II	VAMP I/II	Q76F
BoNT/C1	Unknown	Syntax. 1A/B SNAP-25	K254A, K233A R198A
BoNT/D	SV2 A, B, C	VAMP I/II	K61L, K59L
BoNT/E	SV2 A, B	SNAP-25	R180I
BoNT/F	SV2 A, B, C	VAMP I/II	Q60K, Q58K
BoNT/G	Syt - I/II	VAMP I/II	A81A; A83A
TeTx	SV2 Nidogen 1/2	VAMP I/II	Q76F

Trends in Molecular Medicine

Figure 2. Molecular Premise for Neuron Specificity and Activity-Dependent Uptake of Clostridial Neurotoxins (CNTs). (A) Crystal structure of botulinum neurotoxin type A (BoNT/A) (top). The C-terminal binding domain (H_C) adopts a β-trefoil fold to bind the membrane surface of neurons while the N-terminal jelly-roll motif is proposed to interact with the translocation domain (H_N). The translocation domain is represented by a pair of long helices reminiscent of a viral coiled-coil motif and a long loop (or translocation belt) that wraps around and protects the catalytically active site. The light chain (LC) contains the conserved HExxH motif characteristic of zinc-dependent proteases. Structural comparison of the H_Cs of BoNT/A and tetanus toxin (TeTx) reveals architectural similarities (bottom). Adapted, with permission, from [71]. (B) Summary table of putative neuronal acceptors, target soluble NSF-attachment protein receptors (SNAREs), and scissile bonds for the seven BoNT serotypes plus TeTx.

transport to reach the **perikaryon** of MNs in the spinal cord and from there passes trans-synaptically into inhibitory synapses formed by **glycine- and GABA-ergic interneurons**. Such distinct mechanisms of action are responsible for the truly opposite effects of BoNTs and TeTx on motor functions, causing **flaccid** versus **spastic paralysis**, respectively.

interneurons forming monosynaptic connections with MNs.
Internalization: uptake of biomaterials and reporters by neurons; can be associated with the synaptic vesicle cycle.
Nanovehicles: molecular carriers used for payload targeting and delivery.
Neurotropism: specific affinity of biomolecules for nervous tissue and nerves at the periphery.
Perikaryon: the bulbous compartment of a neuron containing the cell body and nucleus.
Retrograde transport: intra-axonal migration of molecules from the axon terminals to the cell body of neurons.
Soluble NSF-attachment protein receptors (SNAREs): a large family of proteins involved in membrane transport and synaptic vesicle fusion during exocytosis.
Synaptic vesicle cycle: a fundamental, multistep biological process enabling the fusion of synaptic vesicles and neurosecretion followed by recovery of the membrane with the production of a new set of synaptic vesicles for fusion.
Theranostics: a form of diagnostic testing employed to select targeted therapy.

Box 2. Neurotransmitter Release Mechanisms at the NMJ

Chemical transmission at the NMJ is mediated by acetylcholine release from the presynaptic terminals of MNs and activation of nicotinic receptors on the postsynaptic membrane of muscle fibers. Acetylcholine is synthesized in the neuronal cytosol from choline and acetyl-CoA and is stored in synaptic vesicles [72,73]. The accumulation of acetylcholine in the lumen of synaptic vesicles is mainly driven by the electrochemical proton gradient generated by the vesicular ATPase proton pump, which is located in the synaptic vesicle membrane.

Forming a reserve pool, acetylcholine-loaded synaptic vesicles progress to engage with the protein scaffolds of presynaptic release sites, known as active zones, in a process called docking [74,75]. The subsequent priming step, which involves two synaptic vesicle proteins (VAMP, also known as synaptobrevin, and Syt), two proteins in the presynaptic membrane (SNAP-25 and syntaxin), and several soluble cytosolic proteins (most importantly, complexin and Munc18), prepares the synaptic vesicle for rapid and regulated fusion with the presynaptic membrane in response to the rise of intracellular Ca^{2+} [14,76].

Depolarization of the nerve terminal by action potentials results in the opening of membrane Ca^{2+} channels with the influx of Ca^{2+} ions triggering the fusion of primed synaptic vesicles. In response to the fast Ca^{2+} rise and binding to Syt, VAMP forms a coiled-coil complex with SNAP-25 and syntaxin (known also as the SNARE complex), driving membrane fusion with acetylcholine release into the synaptic cleft. This process is tightly controlled by complexin, Munc18, and other regulator proteins [14,17,77]. Acetylcholine diffused out of the synaptic terminals binds to postsynaptic nicotinic receptors, inducing depolarization of muscle fibers and contraction.

During acetylcholine release, the lumen of the synaptic vesicle is briefly exposed to the extracellular fluid, a step critical for high-affinity binding of CNTs to synaptic proteins and their internalization into the nerve terminal by endocytosis. Transient opening of the synaptic lumen also provides a window for the entry of neurotrophic viruses and pathogens as well as targeted delivery of therapeutics using CNT nanocarriers. At the NMJ, most of the fused synaptic membrane is internalized by a clathrin-dependent process [78,79]. Uncoating of synaptic vesicles after endocytosis prepares them for the next cycle through refilling, docking, and priming for exocytosis.

Molecular Basis of BoNT- and TeTx-Mediated MN Targeting

Recognition and anchoring of BoNTs and TeTx to nerve endings are thought to be essential not only for their superb **neurotropism** but also for their fulminant toxicity [18,21]. These processes involve multipart molecular interactions of toxins with membrane glycolipids and synaptic vesicle proteins at the NMJ, followed by a complex intracellular journey. According to the **dual-acceptor model of CNT binding to nerve terminals** [22], the initial attachment of CNTs to **gangliosides** on the surface membrane of nerve terminals is followed by high-affinity binding with specific receptor proteins that facilitate their **internalization** and sorting to various intracellular compartments. Lipid rafts on the cell membrane enriched with various gangliosides (GD1a, GD1b, and GT1b), glycoproteins, and disialocarbohydrates have been proposed to facilitate the initial association and enrichment of CNTs at presynaptic terminals, followed by more avid binding to synaptic protein receptors. Both the kinetics and the pharmacodynamics of the binding of neurotoxins to nerve terminals have been described in great detail in various models, including nonhuman primates [23–25]. Given the enrichment of synaptic vesicles at presynaptic elements of NMJs and the association of CNT toxicity with neurosecretory activity, synaptic vesicle proteins have been interrogated and proven to be high-affinity receptors of CNTs [26–28]. The propensity of BoNTs to dwell at NMJs while TeTx migrates retrogradely to MNs and to inhibitory neurons and synapses (further upstream) in the CNS render these two classes of toxins an attractive tool for targeting/delivering therapeutics to various MN compartments and synapses (peripheral versus central). These putative neuron-targeting and delivery probes are the subject of ongoing research and recent preclinical studies.

Criteria for Activity-Dependent Internalization of Drug-Loaded Atoxic CNTs

As indicated above, the low-affinity interaction of CNTs with the lipids of surface membranes is followed by their more avid binding to synaptic vesicle proteins. The notion of dual CNT receptors has prompted intense research into the molecular mechanisms facilitating the initial steps of BoNT and TeTx toxicity [29,30]. The first indications of membrane proteins acting as CNT receptors were provided by reports showing protease sensitivity of BoNT binding to synaptosomes in rats [31,32]. These discoveries propelled follow-up studies into the role of protein–protein interactions in the internalization of BoNTs in association with synaptic vesicles in murine models; these patterns manifested as greater intoxication of nerve terminals undergoing intense activity [20,21,33]. The concept of synaptic vesicle proteins as BoNT receptors received strong validation from *in vitro* crosslinking and biochemical studies showing, for the first time that the intraluminal domain of the synaptic protein synaptotagmin (Syt) is critical for the toxicity of BoNT/B in rats [27,34]. As a principal Ca^{2+} sensor for regulated neurotransmitter release, Syt turnover at NMJs and other synapses was found to be coupled to exocytosis and then **endocytosis**. Further research on the role of various Syt isoforms confirmed the key function of Syt-I/II in the internalization and toxicity of BoNT/B [35]. Using protein pull-down experiments, site-directed mutagenesis, and molecular mapping, 20 juxtamembrane amino acids of the luminal N-terminal domain of Syt have been subsequently identified as the intraluminal BoNT/B-binding motif in mice [26]. The same Syt-I/II domain was later reported to serve as a high-affinity binding site for BoNT/G [36]. These discoveries were followed by a series of reports expanding the list of synaptic vesicle proteins serving as receptors for various BoNT serotypes in different vertebrates (Figure 2B). In the case of BoNT/A, the H_C domain binds to several isoforms of the synaptic vesicle protein SV2 [37,38]. Interaction of these BoNT serotypes with SV2 occurs at the level of a conserved loop located within the lumen of the synaptic vesicle. Similar to Syt, the intraluminal loop of SV2, which is transiently exposed on the surface of neurons on synaptic vesicle exocytosis [39–41] presumably becomes accessible to BoNT/A, facilitating its uptake. In murine models, BoNT/A has been shown to interact with a 125-residue luminal segment of SV2 [42], with the SV2C isoform displaying the highest affinity for BoNT/A followed by SV2A and SV2B [43]. Different isoforms and post-translational variants of SV2 can also bind to BoNT/D, E, and F and promote their entry into neurons [37,44,45]. This leaves only the protein receptor for

BoNT/C to be identified. The fact that BoNTs infiltrate neurons by exploiting the **synaptic vesicle cycle** not only provides grounds for their remarkable neuron selectivity but also renders them effective in targeting specific processes unfolding within synaptic terminals. Delivery of restorative cargo and therapeutics with localized synaptic effects might thus be envisioned.

The Dilemma of TeTx Receptors: Peripheral versus Central Synapses

Despite fundamental structural similarities and common entry sites, the BoNT- and TeTx-induced antagonistic paralytic effects are largely attributed to the differences in their post-endocytosis fate and toxicity sites. Unlike strictly synaptic vesicle-coupled internalization of BoNTs (detailed above), the relationship between synaptic activity and the uptake of TeTx by neurons varies depending on the model used [9,46,47]. Importantly, the initial anchoring of TeTx to nerve terminals, similar to that of BoNTs, entails loose attachment to glycolipids including GD1b and GT1b as well as glycophosphatidylinositol (GPI)-anchored glycoproteins at membrane surfaces [48–50]. In nerve growth factor-differentiated PC-12 cells, the GPI-anchored glycoprotein Thy-1 has been shown to serve as the high-affinity receptor for TeTx [51], while in rat primary MNs in culture TeTx H_C shares retrograde transport organelles with the low-affinity p75 neurotrophin receptor ($p75^{NTR}$) [52]. In murine MNs TeTx has been proposed to make use of retrograde transport organelles with physiological ligands, such as neurotrophins, that after entry into neurons escape the acidifying presynaptic compartments for long-range retrograde transport. Contrary to the canonical clathrin-dependent route of membrane recovery implicated in regulated exocytosis and BoNT uptake, the clathrin-, dynamin-, and AP-dependent uptake of TeTx H_C do not involve the adaptor protein epsin-1, which is known to facilitate synaptic vesicle budding during endocytosis [53]. As a result, and in contrast to BoNTs sorted to v-ATPase-mediated acidified endosomes, TeTx is channeled to non-acidified compartments followed by loading onto retrograde carriers [53,54]. The latter are enriched with the small GTPase Rab7 and low- or high-affinity neurotrophin receptors (p75NTR and TrkB, respectively), which are destined for fast retrograde transport to the CNS [53,55]. Nevertheless, recent evidence has shown that the synaptic SV2 protein is required for both uptake and toxicity of TeTx in rat cultured neurons [46,47], while at the NMJ the extracellular matrix protein nidogen has been identified as the receptor for the specific binding of TeTx H_C and TeTx in mice [56]. These findings suggest that various high-affinity receptors for TeTx may exist at the NMJ and central synapse and that they may have differential post-internalization fates in central versus motor neurons [47]. Unlike the intracellular journey of BoNTs coupled to the synaptic vesicle cycle, TeTx emerges as flexible and capable of using various entry routes and sorting pathways. At the NMJ, it can target compartments destined for retroaxonal transport, while at central synapses it seems to prefer acidifying endosomes, a critical step for the translocation of the LC protease at synaptic terminals impinging on MNs. These striking differences between BoNTs and TeTx not only are important for understanding the cellular mechanisms of their paralytic effects but also should be considered for cargo selection and targeting specific neuronal compartments and functions when designing future therapeutics. The presynaptic secretory machinery of NMJs, for instance, is expected to be more amenable to payload tethered to BoNTs, while the use of TeTx is more appropriate for retroaxonal delivery of therapeutics to MNs and upstream synapses and neurons.

Partnership Bound for Superb Performance: Implications for Cargo Delivery

Over past decades, the HC and **C-terminal fragments (CTFs)** of CNTs have been considered not only as the sole determinants of their potent neurotropism but also as key factors determining their post-endocytic fate within cells. The first report of the cytoplasmic delivery of TeTx LC by BoNT/A HC in isolated mouse phrenic nerve hemidiaphragm preparations [57] was followed by demonstrations of enhanced transfer of peptides and reporters to primary neurons and cells using CNT-binding fragments *in vitro* [58–62]. However, the extent of the translocation of HC- or H_C-tethered cargoes into the cytoplasm of target cells has remained unclear, implying an active contribution of the H_N translocation domain in the cytoplasmic delivery of the LC with

toxicity. The notion of close cooperation between various CNT domains for maximal performance was supported by the demonstration that a protease-inactive mutant of full-length TeTx (TeTIM) was superior to HC and H_C in antagonizing the paralytic effects of TeTx in mice *in vivo* [63]. Results from follow-up studies of molecular cargo or genetic material delivery with BoNT/D, detoxified full-length BoNTs (BoTIMs), or TeTx (TeTIM and TeTx fragments) fused with **core streptavidin (CS)** supported this premise, demonstrating their superior performance in neuron binding, internalization, and intracellular transport compared with the binding fragments [8,9,59,64]. Comparison of the performance of CS-TeTIM with fusion proteins comprising CS with TeTx HC, H_C, or H_{CC} showed that, in both neuron-binding and internalization assays, TeTIM outperformed TeTx CTF fragments [9]. Innocuous CS-TeTIM also proved superior to CS-TeTx in counteracting TeTx-induced cleavage of VAMP-2 in rat cultured neurons, while *in vivo* CS-TeTIM uptake and mobility in MN terminals as well as its deposition in spinal cord neurons was higher than that of CS-TeTx-H_C [9]. Evidence from studies of chimeric CNTs further supports a close synergistic partnership of different domains, suggesting a key role for LC–HC interactions in determining the site of the paralytic effects of CNTs. Through exchange of the acceptor-binding HC moieties between BoNTs (A and E) and TeTx in rats, local delivery of BoNT/A and BoNT/E proteases at NMJs and retrograde transport to the CNS were shown not be determined by the HC or H_C alone but rather involved close interactions with the LC [65]. Overall, it appears that the productive toxicity and superb performance of CNTs as vehicles for neuron targeting and delivery of therapeutics require intimate cooperation between different domains of CNTs and thus these toxin domains might offer flexibility in facilitating the delivery of biomolecules to various compartments of neurons.

Targeting and Retroaxonal Transfer of Therapeutic Vectors to MNs

The reports reviewed above not only testify to the incompleteness of our current understanding of the biology of these deadly toxins but also endorse further interrogation of detoxified full-length CNTs for neuron targeting and therapeutic delivery. While informative as model carriers for neurobiological studies, C-terminal binding fragments of CNTs have proved disappointing as delivery vehicles in preclinical trials, with unsatisfactory performance in murine models [66,67]. By contrast, CS-BoTIM/B has rather high efficiency in the transfer of small biocargo and fluorescence reporters to primary MNs *in vitro* or NMJs *ex vivo* (murine) [64,68]. Likewise, transduction of the therapeutic *t-SNAP25* gene or a reporter GFP using CS-BoTIM/B- or CS-TeTIM-targeted lentiviral vectors was highly efficient in rat primary cultured neurons and MNs *in vivo* [8,9,64] (Box 3). Comparative analysis with biochemical and electrophysiological assays showed that full detoxified CNTs outperformed the CS-fused binding fragments in both cargo and gene delivery [9,64,68] (Figure 3). Resistant to three BoNTs (BoNT/A, C, and E), a t-SNAP-25 protein harboring D179K, M182T, and R198T point mutations at scissile bonds showed significantly higher tolerance to BoNT/A and BoNT/E in rat MNs, with a capacity to support synaptic functions under challenge by toxins [9,64]. Notably, the total amount of expressed SNAP-25 in neurons transduced with a CS-BoTIM/B-targeted *t-SNAP25* gene remained unaltered, suggesting that functional protection by the toxin-resistant SNAP-25 was achieved rather than partial rescue due to enhanced SNAP-25 quantities in the same setting [64]. The t-SNAP-25 protein, however, was ineffective against BoNT/C1 owing to BoNT/C1's ability to arrest synaptic release by cleaving another ubiquitous SNARE, syntaxin [64]. Of note, the protective effect of CS-TeTIM-targeted *t-SNAP25* in mice *in vivo* was limited to MNs and their excitatory synaptic inputs [9,69]; unlike unaffected spontaneous EPSCs, BoNT/A strongly suppressed the inhibitory synaptic inputs of MNs [9]. By contrast, TeTx-binding fragments in the same study failed to enable the expression of functional t-SNAP-25 in MNs. These observations suggest that MN targeting and retroaxonal delivery of cargo or therapeutic genes with full-length detoxified TeTx might exceed that of its binding fragments but remains limited to MNs, despite the well-recognized trans-synaptic toxicity of TeTx.

 CellPress

Box 3. Vectors Used for Therapeutic Targeting of MNs

Therapeutic SNAP-25 gene (*t-SNAP-25*)

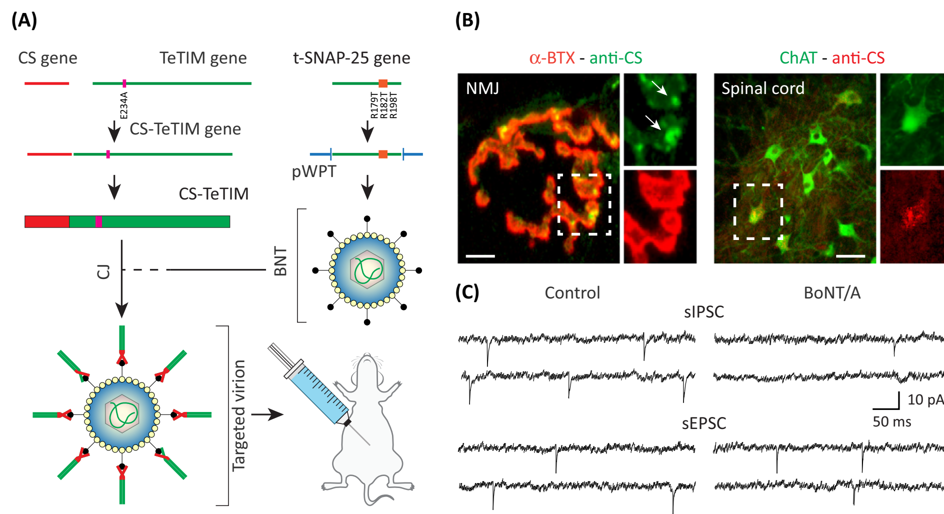
t-SNAP-25 production entailed the cloning of a His_6-tagged mutated *SNAP-25* gene sequence into a pWPT vector. His_6-S25-R198T already resistant to BoNT/A [64] was rendered additionally nonsusceptible to cleavage by BoNT/E or BoNT/C1 by the introduction of two further mutations: D179K and M182T. The lentivirus was biotinylated with EZ-link sulfo-NHS-SS-biotin, conjugated to CS-BoTIM/B, and purified using the His_6 tag.

CS-BoTIM/B

pQE60 containing the *CS* gene was restriction digested with *Bgl*II and ligated into pET-29A encoding full-length BoNT/B that had been codon optimized for *Escherichia coli* with a C-terminal His_6 tag incorporated. Two mutations were introduced into the BoNT/B metalloprotease site: E231A and H234Y. After sequence confirmation, the CS-BoTIM/B-His_6 was expressed in *E. coli*, purified, and nicked, yielding a di-chain used for viral targeting.

CS-TeTIM and CS-TeTx

CS vector was amplified and subcloned into pET29A, generating CS-pET. TeTIM was produced by E234A mutagenesis of the TeTx gene. PCR products were self-ligated and transformed into *E. coli* TOP10. DNA fragments were amplified with forward primers [8] for TeTIM and H_C(TeTx). CS-TeTIM and CS-H_C(TeTx) genes were expressed in *E. coli*, purified, and nicked, yielding a di-chain used for viral targeting.



Trends in Molecular Medicine

Figure 3. A Platform for Targeting the *t-SNAP-25* Gene to Motor Neurons (MNs) with Functional Validation. (A) Schematic of core streptavidin (CS)-TeTIM tailored for targeted delivery of the *t-SNAP-25* gene to rat spinal cord MNs (left). CS-TeTIM was produced through the fusion of CS and detoxified tetanus toxin (TeTx) (E234A point mutation) genes (left); a triply mutated (R179T, R182T, and R198T) *t-SNAP-25* gene was cloned in a pWPT lentiviral vector (right). After biotinylation (BNT) of lentiviral particles and conjugation (CJ) to CS-TeTIM, the targeted material was injected into rat tongue or forelimb triceps muscle. (B) Representative confocal microscopy images of CS at the neuromuscular junction (NMJ) (left) and spinal cord MNs (right) of the rat. NMJs were visualized by injection of rhodamine-labeled α-BuTX in the lateral quadrant of the tongue. The presence of CS in NMJs (white arrows) was detected 8 h after injection of CS-H_C(TeTx) or CS-TeTIM in the same location, by immunohistochemistry. Antistreptavidin monoclonal antibody followed by AlexaFluor 594-labeled secondary antibody was used for detection of CS in NMJs while its presence in MNs of the cervical spinal cord enlargement was verified with double staining using the MN marker choline acetyltransferase (ChAT). AlexaFluor 488-labeled secondary antibody was used to develop ChAT staining and for visualization of MNs. Small inset pairs show enlarged segments of the NMJ or a single representative MN in green and red channels. Bar, 30 μm. (C) Typical inhibitory and excitatory post-synaptic currents (IPSCs and EPSCs) recorded in cervical MNs from control or botulinum neurotoxin type A (BoNT/A)-treated spinal cord slices of rat expressing CS-TeTIM-targeted *t-SNAP-25* gene (6 weeks post-injection of targeted viral particles). Note that the frequency of EPSCs is unaffected by BoNT/A treatment. Adapted, with permission, from [9].

Concluding Remarks

In defense of his unorthodox use of mercury, opium, and other potentially dangerous medical approaches, the irascible 16th-century physician Paracelsus wrote 'All things are poison, and nothing is without poison: the dose alone makes a thing not poison' [70]. Over the centuries many of his radical ideas have found wide acceptance, with some laying the foundation of modern medicine. The recent turn in the tide regarding studies of CNTs and their recognition as agents of great therapeutic potential and medical use is yet another milestone in the history of biomedical research testifying to the fluidity of the borderlines between poisons and medications. The ability of BoNTs to subdue hyperactive secretory cells and relax tense muscles has already found extensive applications in medicine. Enhanced delivery of biomolecules and vectors to neurons using CNTs is now taking the spotlight in experimental and preclinical studies, with increasing evidence advocating the use of their detoxified variants. While legitimate concerns remain over the risks related to residual toxicity, recent evidence from animal models with advances in recombinant technologies inspire optimism in the future use of innocuous CNTs as nanocarriers for the transfer of restorative cargo and gene therapies to neurons and synapses. With an expansion in the list of therapeutic gene candidates and small molecules, there is a pressing need for an improvement in tools for neuron targeting and delivery to MNs (see Outstanding Questions and Box 1). In meeting many of these important characteristics, innocuous CNTs might facilitate future interventions in a variety of neurological and neurodegenerative diseases, such as amyotrophic and primary lateral scleroses and various forms of progressive neuromuscular atrophy and palsy as well as certain bacterial and viral pathologies affecting the biology and function of MNs, with potential therapeutic benefits.

Acknowledgments

This work was supported by the Programme for Research in Third Level Institutions (PRTLI) Cycle 4 from the Higher Education Authority of Ireland and the Neuroscience Section Grant for Target-Driven Therapeutics and Theranostics Research (S.V.O. and J.O.D.).

Supplementary data

Supplemental information associated with this article can be found online at doi:10.1016/j.molmed.2016.09.004.

Outstanding Questions

What are the key determinants of the differential specificity and toxicity of the seven BoNT serotypes and TeTx? Despite fundamental similarities and a common entry site into MNs, major differences exist with regard to potency, longevity of paralytic effects, and toxicity sites.

Is the diversity of protein receptors for CNTs of any relevance in terms of their preference for different neuron types or functions? Emerging evidence suggests that various BoNT serotypes and TeTx exhibit selectivity for different (i.e., sensory, motor, autonomic) neuron types and have distinct local versus distal effects after NMJ entry. The functional relevance of molecular differences between CNTs for differential toxicity and therapeutic utility should be demonstrated.

Does the outperformance by detoxified full-length TeTx of binding fragments as a neuron-targeting and delivery vehicle also apply to full atoxic BoNTs? Reports show that detoxified TeTx is superior to C-terminal binding fragments in terms of internalization, retroaxonal transport, and gene delivery to MNs. Can innocuous full-length BoNTs outperform CTFs?

How virions targeted with detoxified TeTx escape endocytic compartments and release genetic material to initiate transduction? Targeted with TeTx, virions are likely to be sorted to non-acidifying compartments predisposed to retrograde transport. In this context, at which stage is genetic material released from transport endosomes to initiate cellular transduction and translation of a protein of interest?

What impact does conjugation of viral particles or small biomolecules have on neurotropism, internalization, and retroaxonal transport of detoxified CNTs? How are these effects comparable with those on C-terminal binding fragments?

What cellular and molecular processes limit the transduction of CS-TeTIM-targeted viral vectors to MNs, averting the trans-synaptic transfer of genetic material into upstream interneurons? Are such constraints specific to the genetic material alone or do they also apply to small biomolecules and other cargo?

References

1. Kim, K.S. (2008) Mechanisms of microbial traversal of the blood–brain barrier. *Nat. Rev. Microbiol.* 6, 625–634
2. McCall, R.L. *et al.* (2014) Pathogen-inspired drug delivery to the central nervous system. *Tissue Barriers* 2, e944449
3. Doran, K.S. *et al.* (2013) Concepts and mechanisms: crossing host barriers. *Cold Spring Harb. Perspect. Med.* 3, a010090
4. Salinas, S. *et al.* (2010) A hitchhiker's guide to the nervous system: the complex journey of viruses and toxins. *Nat. Rev. Microbiol.* 8, 645–655
5. Ribet, D. and Cossart, P. (2015) How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes Infect.* 17, 173–183
6. Beier, K.T. *et al.* (2011) Anterograde or retrograde transsynaptic labeling of CNS neurons with vesicular stomatitis virus vectors. *Proc. Natl Acad. Sci. U.S.A.* 108, 15414–15419
7. Hislop, J.N. *et al.* (2014) Rabies virus envelope glycoprotein targets lentiviral vectors to the axonal retrograde pathway in motor neurons. *J. Biol. Chem.* 289, 16148–16163
8. O'Leary, V.B. *et al.* (2013) Improved lentiviral transduction of ALS motoneurons *in vivo* via dual targeting. *Mol. Pharm.* 10, 4195–4206
9. Ovsepian, S.V. *et al.* (2015) Internalization and retrograde axonal trafficking of tetanus toxin in motor neurons and trans-synaptic propagation at central synapses exceed those of its C-terminal-binding fragments. *Brain Structure Funct.* 220, 1825–1838
10. Patel, M.M. *et al.* (2009) Getting into the brain: approaches to enhance brain drug delivery. *CNS Drugs* 23, 35–58
11. Reardon, T.R. *et al.* (2016) Rabies virus CVS-N2c(DeltaG) strain enhances retrograde synaptic transfer and neuronal viability. *Neuron* 89, 711–724
12. Dolly, J.O. *et al.* (2009) Neuro-exocytosis: botulinum toxins as inhibitory probes and versatile therapeutics. *Curr. Opin. Pharmacol.* 9, 326–335
13. Murray, L.M. *et al.* (2010) Review: neuromuscular synaptic vulnerability in motor neurone disease: amyotrophic lateral sclerosis and spinal muscular atrophy. *Neuropathol. Appl. Neurobiol.* 36, 133–156
14. Sudhof, T.C. (2014) The molecular machinery of neurotransmitter release (Nobel lecture). *Angew. Chem.* 53, 12696–12717
15. Caleo, M. and Schiavo, G. (2009) Central effects of tetanus and botulinum neurotoxins. *Toxicon* 54, 593–599
16. Montecucco, C. and Schiavo, G. (1994) Mechanism of action of tetanus and botulinum neurotoxins. *Mol. Microbiol.* 13, 1–8
17. Ovsepian, S.V. and Dolly, J.O. (2011) Dendritic SNAREs add a new twist to the old neuron theory. *Proc. Natl Acad. Sci. U.S.A.* 108, 19113–19120
18. Schiavo, G. *et al.* (2000) Neurotoxins affecting neuroexocytosis. *Physiol. Rev.* 80, 717–766
19. Schiavo, G. *et al.* (1994) Tetanus and botulinum neurotoxins are zinc proteases specific for components of the neuroexocytosis apparatus. *Ann. N. Y. Acad. Sci.* 710, 65–75
20. Black, J.D. and Dolly, J.O. (1987) Selective location of acceptors for botulinum neurotoxin A in the central and peripheral nervous systems. *Neuroscience* 23, 767–779

21. Dolly, J.O. *et al.* (1984) Acceptors for botulinum neurotoxin reside on motor nerve terminals and mediate its internalization. *Nature* 307, 457–460
22. Montecucco, C. (1986) How do tetanus and botulinum toxins bind to neuronal membrane. *Trends Biochem. Sci.* 11, 314–317
23. Humeau, Y. *et al.* (2000) How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie* 82, 427–446
24. Simpson, L.L. (1986) Molecular pharmacology of botulinum toxin and tetanus toxin. *Annu. Rev. Pharmacol. Toxicol.* 26, 427–453
25. Dolly, J.O. *et al.* (2014) Molecular aspects of botulinum neurotoxin. *Curr. Top. Neurotox.* 4, 9–33
26. Dong, M. *et al.* (2003) Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. *J. Cell Biol.* 162, 1293–1303
27. Nishiki, T. *et al.* (1994) Identification of protein receptor for *Clostridium botulinum* type B neurotoxin in rat brain synaptosomes. *J. Biol. Chem.* 269, 10498–10503
28. Verderio, C. *et al.* (2006) Entering neurons: botulinum toxins and synaptic vesicle recycling. *EMBO Rep.* 7, 995–999
29. Brunger, A.T. and Rummel, A. (2009) Receptor and substrate interactions of clostridial neurotoxins. *Toxicon* 54, 550–560
30. Montal, M. (2010) Botulinum neurotoxin: a marvel of protein design. *Annu. Rev. Biochem.* 79, 591–617
31. Dolly, J.O. *et al.* (1982) Localization of sites for ^{125}I-labelled botulinum neurotoxin at murine neuromuscular junction and its binding to rat brain synaptosomes. *Toxicon* 20, 141–148
32. Kitamura, M. (1976) Binding of botulinum neurotoxin to the synaptosome fraction of rat brain. *Naunyn Schmiedebergs Arch. Pharmacol.* 295, 171–175
33. Simpson, L.L. (2004) Identification of the major steps in botulinum toxin action. *Annu. Rev. Pharmacol. Toxicol.* 44, 167–193
34. Nishiki, T. *et al.* (1993) Solubilization and characterization of the acceptor for Clostridium botulinum type B neurotoxin from rat brain synaptic membranes. *Biochim. Biophys. Acta* 1158, 333–338
35. Kozaki, S. *et al.* (1998) Characterization of *Clostridium botulinum* type B neurotoxin associated with infant botulism in Japan. *Infect. Immun.* 66, 4811–4816
36. Rummel, A. *et al.* (2004) Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. *J. Biol. Chem.* 279, 30865–30870
37. Dong, M. *et al.* (2008) Glycosylated SV2A and SV2B mediate the entry of botulinum neurotoxin E into neurons. *Mol. Biol. Cell* 19, 5226–5237
38. Yao, G. *et al.* (2016) N-linked glycosylation of SV2 is required for binding and uptake of botulinum neurotoxin A. *Nat. Struct. Mol. Biol.* 23, 656–662
39. Karalewitz, A.P. *et al.* (2010) Identification of a unique ganglioside binding loop within botulinum neurotoxins C and D-SA. *Biochemistry* 49, 8117–8126
40. Strotmeier, J. *et al.* (2010) Botulinum neurotoxin serotype D attacks neurons via two carbohydrate-binding sites in a ganglioside-dependent manner. *Biochem. J.* 431, 207–216
41. Zhang, Y. *et al.* (2010) High-level expression, purification, crystallization and preliminary X-ray crystallographic studies of the receptor-binding domain of botulinum neurotoxin serotype D. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 66, 1610–1613
42. Mahrhold, S. *et al.* (2006) The synaptic vesicle protein 2C mediates the uptake of botulinum neurotoxin A into phrenic nerves. *FEBS Lett.* 580, 2011–2014
43. Dong, M. *et al.* (2006) SV2 is the protein receptor for botulinum neurotoxin A. *Science* 312, 592–596
44. Fu, Z. *et al.* (2009) Glycosylated SV2 and gangliosides as dual receptors for botulinum neurotoxin serotype F. *Biochemistry* 48, 5631–5641
45. Peng, L. *et al.* (2011) Botulinum neurotoxin D uses synaptic vesicle protein SV2 and gangliosides as receptors. *PLoS Pathog.* 7, e1002008
46. Blum, F.C. *et al.* (2012) Tetanus toxin and botulinum toxin a utilize unique mechanisms to enter neurons of the central nervous system. *Infect. Immun.* 80, 1662–1669
47. Yeh, F.L. *et al.* (2010) SV2 mediates entry of tetanus neurotoxin into central neurons. *PLoS Pathog.* 6, e1001207
48. Herreros, J. *et al.* (2000) Tetanus toxin fragment C binds to a protein present in neuronal cell lines and motoneurons. *J. Neurochem.* 74, 1941–1950
49. Herreros, J. and Schiavo, G. (2002) Lipid microdomains are involved in neurospecific binding and internalisation of clostridial neurotoxins. *Int. J. Med. Microbiol.* 291, 447–453
50. Munro, P. *et al.* (2001) High sensitivity of mouse neuronal cells to tetanus toxin requires a GPI-anchored protein. *Biochem. Biophys. Res. Commun.* 289, 623–629
51. Herreros, J. *et al.* (2001) Lipid rafts act as specialized domains for tetanus toxin binding and internalization into neurons. *Mol. Biol. Cell* 12, 2947–2960
52. Lalli, G. and Schiavo, G. (2002) Analysis of retrograde transport in motor neurons reveals common endocytic carriers for tetanus toxin and neurotrophin receptor p75NTR. *J. Cell Biol.* 156, 233–239
53. Deinhardt, K. *et al.* (2006) Tetanus toxin is internalized by a sequential clathrin-dependent mechanism initiated within lipid microdomains and independent of epsin 1. *J. Cell Biol.* 174, 459–471
54. Bomba-Warczak, E. *et al.* (2016) Interneuronal transfer and distal action of tetanus toxin and botulinum neurotoxins A and D in central neurons. *Cell Rep.* 16, 1974–1987
55. Bohnert, S. and Schiavo, G. (2005) Tetanus toxin is transported in a novel neuronal compartment characterized by a specialized pH regulation. *J. Biol. Chem.* 280, 42336–42344
56. Bercsenyi, K. *et al.* (2014) Tetanus toxin entry. Nidogens are therapeutic targets for the prevention of tetanus. *Science* 346, 1118–1123
57. Weller, U. *et al.* (1991) Cooperative action of the light chain of tetanus toxin and the heavy chain of botulinum toxin type A on the transmitter release of mammalian motor endplates. *Neurosci. Lett.* 122, 132–134
58. Andreu, A. *et al.* (2008) *Clostridium* neurotoxin fragments as potential targeting moieties for liposomal gene delivery to the CNS. *Chembiochem* 9, 219–231
59. Bade, S. *et al.* (2004) Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates. *J. Neurochem.* 91, 1461–1472
60. Dobrenis, K. *et al.* (1992) Neuronal lysosomal enzyme replacement using fragment C of tetanus toxin. *Proc. Natl Acad. Sci. U.S.A.* 89, 2297–2301
61. Francis, J.W. *et al.* (2004) Tetanus toxin fragment C as a vector to enhance delivery of proteins to the CNS. *Brain Res.* 1011, 7–13
62. Knight, A. *et al.* (1999) Non-viral neuronal gene delivery mediated by the HC fragment of tetanus toxin. *Eur. J. Biochemistry* 259, 762–769
63. Li, Y. *et al.* (2001) Recombinant forms of tetanus toxin engineered for examining and exploiting neuronal trafficking pathways. *J. Biol. Chem.* 276, 31394–31401
64. O'Leary, V.B. *et al.* (2011) Innocuous full-length botulinum neurotoxin targets and promotes the expression of lentiviral vectors in central and autonomic neurons. *Gene Ther.* 18, 656–665
65. Wang, J. *et al.* (2012) Novel chimeras of botulinum and tetanus neurotoxins yield insights into their distinct sites of neuroparalysis. *FASEB J.* 26, 5035–5048
66. Fishman, P.S. (2009) Tetanus toxin. In *Botulinum Toxin* (Jankovic, J. *et al.*, eds), pp. 406–424, Elsevier
67. Toivonen, J.M. *et al.* (2010) Tetanus toxin C-fragment: the courier and the cure? *Toxins* 2, 2622–2644
68. Edupuganti, O.P. *et al.* (2012) Targeted delivery into motor nerve terminals of inhibitors for SNARE-cleaving proteases via liposomes coupled to an atoxic botulinum neurotoxin. *FEBS J.* 279, 2555–2567
69. Nishimaru, H. *et al.* (2005) Mammalian motor neurons corelease glutamate and acetylcholine at central synapses. *Proc. Natl Acad. Sci. U.S.A.* 102, 5245–5249
70. Paracelsus, T. (1965) *Werke. Bd. 2,* Wissenschaftliche Buchgesellschaft
71. Turton, K. *et al.* (2002) Botulinum and tetanus neurotoxins: structure, function and therapeutic utility. *Trends Biochem. Sci.* 27, 552–558

CellPress

72. Carlson, N.R. (2001) *Physiology of Behavior*. (7th edn), Allyn and Bacon

73. Taylor, P. and Brown, J.H. (1999) *Synthesis, Storage and Release of Acetylcholine,* Lippincott-Raven

74. Harlow, M.L. *et al.* (2013) Alignment of synaptic vesicle macromolecules with the macromolecules in active zone material that direct vesicle docking. *PLoS One* 8, e69410

75. Zhai, R.G. and Bellen, H.J. (2004) The architecture of the active zone in the presynaptic nerve terminal. *Physiology* 19, 262–270

76. Katz, B. and Miledi, R. (1965) The effect of calcium on acetylcholine release from motor nerve terminals. *Proc. Biol. Sci.* 161, 496–503

77. Sudhof, T.C. and Rothman, J.E. (2009) Membrane fusion: grappling with SNARE and SM proteins. *Science* 323, 474–477

78. Di Paolo, G. and De Camilli, P. (2003) Does clathrin pull the fission trigger? *Proc. Natl Acad. Sci. U.S.A.* 100, 4981–4983

79. Wu, M. *et al.* (2010) Coupling between clathrin-dependent endocytic budding and F-BAR-dependent tubulation in a cell-free system. *Nat. Cell Biol.* 12, 902–908