The Elusive Compass of Clostridial Neurotoxins: Deciding When and Where to Go?

Kinga Bercsenyi, Francesco Giribaldi and Giampietro Schiavo

Abstract Axonal transport ensures long-range delivery of essential components and signals between proximal and distal areas of the neuron, and it is crucial for neuronal homeostasis and survival. Several pathogens and virulence factors use this route to gain access to the central nervous system, exploiting the complex and still poorly understood trafficking mechanisms that regulate the dynamics of their cellular receptors. Studying the intracellular transport of neurotropic pathogens is therefore instrumental to glean new insights into these important molecular events. Botulinum (BoNT) and tetanus (TeNT) neurotoxins bind with high affinity to a variety of neurons and are internalised by specialised endocytic pathways leading to specific intracellular fates. Whereas BoNT trafficking is largely confined to the neuromuscular junction, TeNT is internalised in signalling endosomes shared with neurotrophins and their receptors, which are recruited to the fast axonal retrograde transport pathway. Recently, important paradigms regarding the mechanisms by which BoNT and TeNT interact with their cellular targets and are transported in neurons have been challenged. In this review, we summarise new findings concerning the uptake and intracellular trafficking of these neurotoxins, and discuss their implications in terms of the physiological effects of BoNT and TeNT in the central nervous system.

Keywords Axonal transport · Botulinum neurotoxin · Endocytosis motor neurons · Tetanus toxin

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Abbreviations

Aβ	Amyloid beta
AP2	Adaptor protein 2
BAR	Bin–amphiphysin–rvs domain
BDNF	Brain-derived neurotrophic factor
BoNT	Botulinum neurotoxins
CAR	Coxsackie- and adenovirus receptor
CAV2	Canine adenovirus 2
CCP	Clathrin coated pits
CCV	Clathrin coated vesicles
ChAT	Choline acetyltransferase
CHO	Chinese hamster ovary
CME	Clathrin-mediated endocytosis
CNS	Central nervous system
CNT	Clostridial neurotoxins
DRG	Dorsal root ganglia
ERK	Extracellular signal-regulated kinase
GPI	Glycosylphosphatidylinositol
HC	Heavy chain
H_CT	Binding fragment of tetanus toxin
LC	Light chain
Kidins220/ARMS	Kinase-D-interacting substrate of 220 kDa/ankyrin-rich membrane spanning
MT	Microtubules
NMJ	Neuromuscular junction
NT	Neurotrophins
$p75^{NTR}$	p75 neurotrophin receptor
PC12	Pheochromocytoma cells
PLCγ1	Phospholipase Cγ1
PrP	Prion protein
PtdIns(4,5)P_2	Phosphatidylinositol(4,5)bisphosphate
PV	Poliovirus
SC	Superior colliculus
SNAP-25	Synaptosomal associated protein of 25 kDa
SNARE	Soluble NSF attachment protein receptor
SV2	Synaptic vesicle glycoprotein 2
SV40	Simian virus 40
Syt	Synaptotagmin
TeNT	Tetanus neurotoxin
Trk	Tropomyosin-receptor-kinase
VAMP	Vesicle associated membrane protein

Contents

1 Introduction

Efficient mechanisms ensuring the reliable exchange of information between cells and their environment are essential for all organisms. The outer surface of the plasma membrane provides a platform for a wide range of receptors, which sense extracellular signals and inform cells about changes in their surroundings. To ensure that cells receive relevant messages and are able to assemble suitable physiological responses, both the lipid and protein compositions of the plasma membrane must be tightly regulated. A main player in this homeostatic control is the process of endocytosis, in which selective components of the plasma membrane are internalised in a highly regulated fashion and undergo intracellular trafficking leading to degradation, recycling or targeting to other membrane compartments (McMahon and Boucrot 2011). The main roles of endocytosis include nutrient uptake, receptor-mediated signalling and the turnover of membrane components and receptors (Hoeller et al. 2005). Given the key physiological role and evolutionary conservation of these mechanisms, numerous pathogens and virulence factors, including clostridial neurotoxins (CNT) exploit selective endocytic routes to gain access to host cells. Despite the plethora of different endocytic cargoes, surprisingly little information is available regarding the molecular mechanisms regulating the recruitment of ligand-receptor complexes to specific carriers, their internalisation and transport and their ultimate fate. In light of this, infectious agents represent invaluable tools shaped by millions of years of evolution to study the molecular determinants of these membrane trafficking pathways (Schiavo and van der Goot 2001).

The most extensively studied type of endocytosis is clathrin-mediated endocytosis (CME). The list of proteins involved in the regulation of CME has been continuously growing, and as a result, a detailed view of the molecular mechanisms controlling its progression is now available (McMahon and Boucrot 2011). Mechanistically, CME occurs via four main steps, the first of which is the nucleation of

clathrin at specific membrane sites. This process is initiated by accessory and adaptor proteins, such as adaptor protein 2 (AP2) and epsins (Henne et al. 2010; Jackson et al. 2010; Qualmann et al. 2011; Taylor et al. 2011). Following the recruitment of membrane curvature-inducing proteins, clathrin-coated pits (CCPs) are formed. Once the budding is completed, clathrin-coated vesicles (CCV) are clipped off the membrane by dynamin, a GTPase involved in membrane remodelling (Harper et al. 2011; Ferguson and De Camilli 2012), and the clathrin basket is removed by cytoplasmic factors to form an uncoated vesicle (Bocking et al. 2011). Interestingly, CME was shown to be the main, but not the only route taken by CNT to get access to their target cells (Deinhardt et al. 2006a; Montal 2010).

2 Clostridial Neurotoxins are Multi-Domain Proteins

The CNT family comprises tetanus toxin (TeNT) and several related botulinum neurotoxins (BoNT, serotypes A to G; Swaminathan 2011). TeNT and BoNT are synthesised as single chain proteins of 150 kDa, which are cleaved by endogenous or tissue proteases, resulting in a 50 kDa light chain (LC) and a 100 kDa heavy chain (HC) linked via a disulphide bridge. The carboxy-terminal half of the HC is responsible for the binding of CNT to the neuronal surface, whilst the amino-terminal half is involved in the translocation of the LC through the endosomal membrane (Montal 2010). The LC contains the active site of the neurotoxin and displays a very specific metalloprotease activity (Schiavo et al. 2000). To date, the synaptic SNARE (soluble NSF attachment protein receptor) proteins syntaxin-1, SNAP-25 (synaptosomal-associated protein of 25 kDa) and VAMP-1–2 (vesicle-associated membrane protein) (termed also synaptobrevin-1 and -2) are the only identified substrates of TeNT and BoNT (McMahon et al. 1993; Schiavo et al. 2000), in addition to VAMP-3, syntaxin-2 and syntaxin-3. Cleavage of these synaptic SNARE proteins yields the persistent blockade of neurotransmitter release in intoxicated neurons (Schiavo et al. 1992; Blasi et al. 1993a, b; Schiavo et al. 1993a, b; Yamasaki et al. 1994).

CNT share their core structural characteristics (Fig. 1), cleave the same class of intracellular substrates and are taken up by the same neurons (Schiavo et al. 2000). However, once endocytosed, TeNT and BoNT are sorted to different membrane trafficking pathways (Fig. 1). BoNT mainly target the neuromuscular junction (NMJ) in vivo, and only a minor fraction of the toxin is transported back to the cell body of the motor neuron (Restani et al. 2012a). In contrast, TeNT efficiently reaches the inhibitory interneurons located in the spinal cord (Fig. 1). This distinct intracellular trafficking leads to a different symptomatology of the pathologies caused by BoNT and TeNT. Botulism, which is caused by BoNT, leads to flaccid paralysis, whilst tetanus is characterised by a sustained spastic paralysis. It remains to be seen whether or not this difference in sorting is due to specific receptor complexes for TeNT and BoNT, which would target them to distinct endocytic compartments, or to the recruitment of different sorting factors after internalisation.

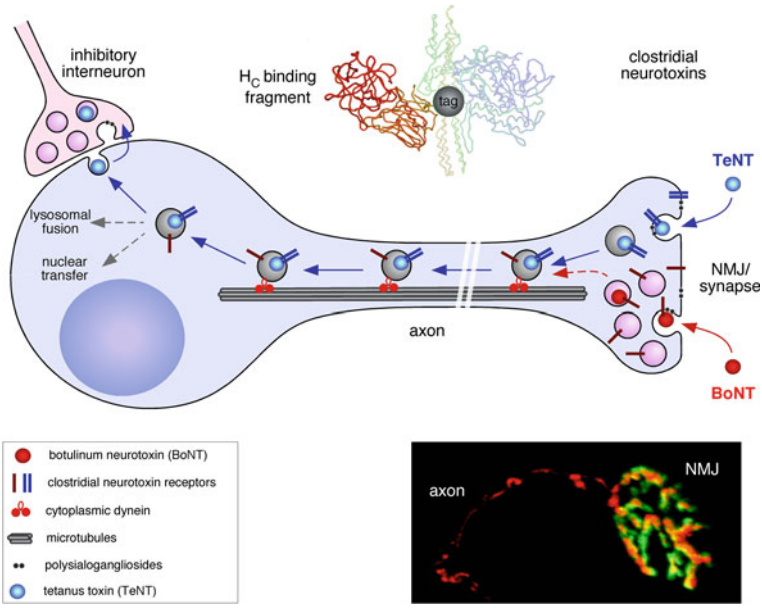

Fig. 1 Trafficking of botulinum and tetanus neurotoxins in neurons. *Top*. The three-domain structure of BoNT/A (Lacy et al. 1998), which is shared with the other CNT, is characterized by a 50 kDa LC containing the active site (shaded, on the *right*) and a 100 kDa HC responsible for membrane translocation (HN; shaded, *centre*) and binding to the neuronal surface (HC; unshaded, on the *left*). CNT fragments can be expressed as recombinant proteins bearing specific short domains that allow their labelling with fluorophores (tag) and other functional groups (Deinhardt et al. 2006b). Due to its binding and sorting characteristics and its lack of toxicity, fluorescent HCs can be used instead of the full length CNT to study binding, transport and transcytosis. *Middle*. Schematic representation of CNT trafficking in a spinal cord motor neuron. TeNT (*light blue*) and BoNT (*red*) specifically bind to neuronal membranes via receptor complex(es) formed by polysialogangliosides (*black dots*) and synaptic proteins (*blue* and *red bars*). CNT are then internalised and sorted into different intracellular compartments. Whilst BoNT remain mainly localised to the NMJ, where they enter the synaptic vesicle recycling pathway, TeNT undergoes fast axonal retrograde transport exploiting MT as tracks (*grey*) and cytoplasmic dynein as a main molecular motor (*red*). Once it has reached the soma, TeNT is trancytosed into inhibitory interneurons, where the LC reaches the cytoplasm and blocks neurotransmitter release. *Bottom*. Mouse NMJ after intramuscular administration of HCT (*red*). HCT is internalised and transported into the motor neuron shaft. The motor endplate has been counterstained with α-bungarotoxin (*green*)

3 Binding of Clostridial Neurotoxins to the Cell Surface

Complex gangliosides were shown to serve as receptors for CNT. TeNT binds to GT1b and GD1b, whereas BoNT bind to GT1b and GD1a (Habermann and Dreyer 1986; Schengrund et al. 1991; Yowler and Schengrund 2004). The affinity of CNT to immobilised polysialogangliosides is in the high nanomolar range, whilst these neurotoxins bind to synaptosomes and neurons with a much higher affinity, making

their interactions with neuronal membrane almost irreversible (Habermann and Dreyer 1986). The addition of exogenous polysialogangliosides to non-neuronal cells, such as undifferentiated rat pheochromocytoma (PC12), renders them sensitive to TeNT or BoNT/A (Marxen and Bigalke 1989; Marxen et al. 1990). In agreement with this finding, neuraminidase treatment makes cells insensitive to CNT by removing sialic acid residues from the plasma membrane (Bigalke et al. 1986; Marxen and Bigalke 1989). These results, together with the partial insensitivity to CNT of mice lacking complex gangliosides (Kitamura et al. 1999, 2005; Rummel et al. 2009), indicate that these lipids are essential components of CNT receptor complexes. On the other hand, the relatively low affinity of polysialogangliosides for TeNT and BoNT and the major inhibitory effect on binding caused by pre-treatment of neurons with extracellular proteases, strongly suggests the existence of a dual protein and lipid receptor for CNT (Montecucco 1986). According to the dual receptor hypothesis (Montecucco 1986; Rummel et al. 2007), polysialogangliosides either favour the recruitment of TeNT and BoNT to specific areas of the plasma membrane enriched in protein receptors, or maintain these toxins in a preferred conformation for the receptor to bind.

The molecular identification of the protein receptors for CNT posed a challenge to the field. Both binding and internalisation of BoNT were shown to be activity-dependent (Black and Dolly 1986; Keller et al. 2004; Baldwin and Barbieri 2007) suggesting that synaptic vesicle proteins might be involved in BoNT binding and uptake. The intraluminal leaflet of the synaptic vesicle membrane becomes transiently exposed to the extracellular medium upon fusion of synaptic vesicles with the active zone during synaptic stimulation, potentially allowing the intravesicular domains of synaptic vesicle proteins to interact with the binding domain of CNT and act as their protein receptors. Pioneering work from Kozaki's group has demonstrated that BoNT/B binds simultaneously to polysialogangliosides and synaptotagmin-I (Syt-I), the main calcium sensor for synaptic vesicle fusion, and the closely related isoform synaptotagmin-II (Syt-II), with ten times higher affinity than polysialogangliosides alone (Ochanda et al. 1986; Nishiki et al. 1994; 1996a). However, direct evidence that the luminal domain of both Syt-I and Syt-II binds BoNT/B and mediates the internalisation of the toxin was provided only a decade later (Dong et al. 2003, 2007). Hippocampal neurons lacking Syt-I are insensitive to both BoNT/B and BoNT/G, but can be rendered sensitive by overexpression of Syt-I or -II (Dong et al. 2007). Similarly, the cytoplasm of PC12 and Chinese hamster ovary (CHO) cells became accessible to BoNT/B upon the surface expression of the intraluminal domain of Syt-I/II (Nishiki et al. 1996b; Pirazzini et al. 2011). However, Syt are not universal protein receptors for BoNT, as only BoNT/B, BoNT/G and the mosaic toxin BoNT/DC bind to Syt-I/II (Rummel et al. 2004, 2007; Peng et al. 2012). Based on the hypothesis that the luminal portion of synaptic vesicle proteins may serve as protein receptor of the remaining BoNT serotypes, two groups independently demonstrated that synaptic vesicle glycoprotein 2 (SV2) acts as a protein receptor for BoNT/A (Dong et al. 2006; Mahrhold et al. 2006) and several other serotypes, such as BoNT/E (Dong et al. 2008), BoNT/F (Rummel et al. 2009) and BoNT/D (Peng et al. 2011).

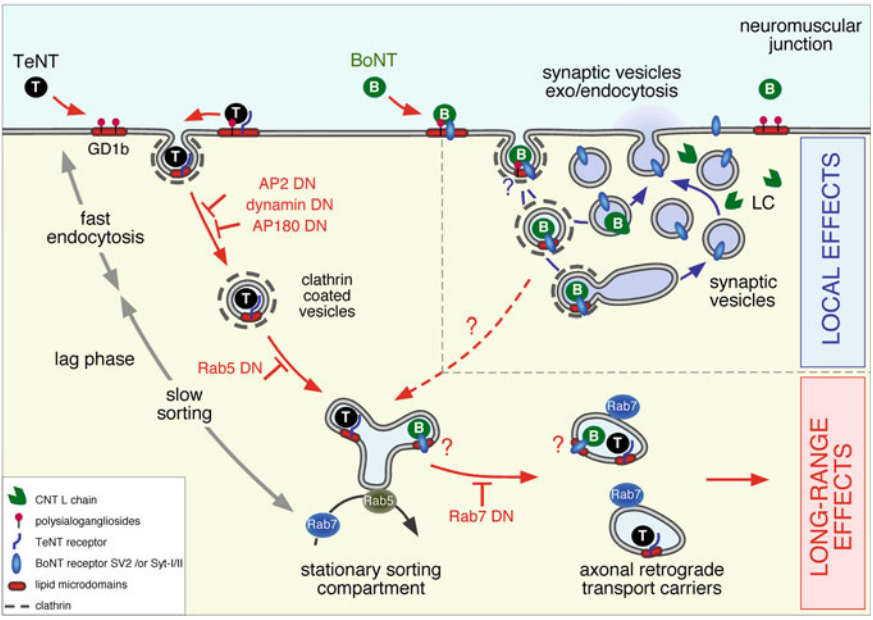

Fig. 2 Uptake of TeNT and BoNTs in cultured motor neurons. Synaptic vesicle exo/endocytosis accounts for the majority of endocytic events at the presynaptic terminal and may involve multiple clathrin-dependent step(s). Several BoNTs exploit this pathway for their internalization into neurons by binding to the luminal domain of specific vesicle proteins, such as SV2 and Syt-I/II. High affinity interaction to these proteins requires polysialogangliosides and may be regulated by lipid microdomains. Once internalised into the synaptic vesicle lumen, acidification driven by the vATPase triggers the translocation of LC into the nerve terminal cytoplasm that leads to the cleavage of SNARE proteins essential for membrane fusion. This process determines the long-lasting inhibition of neurotransmitter release at the NMJ (*local effects*). At very high doses, TeNT enters this pathway and induces a flaccid paralysis similar to that caused by BoNT. In contrast, at physiological doses TeNT exploits a pathway requiring lipid microdomains and the clathrin machinery that is largely independent of synaptic vesicle exo/endocytosis. At the NMJ, TeNT binds to a lipid-protein receptor complex containing polysialogangliosides such as GD1b, and is then laterally sorted into clathrin-coated vesicles. During this sorting event, GD1b is excluded from the toxin receptor complex (Deinhardt et al. 2006a). Internalisation of TeNT is dependent on dynamin, AP-2 and AP180, but does not require epsin1. Once internalised, TeNT is targeted to a stationary (or oscillating) early sorting compartment positive for the small GTPase Rab5, to which other endocytic routes may converge. Some of these routes may be responsible for the entry of specific BoNT serotypes (e.g. BoNT/A) to sorting endosomes, their targeting to the axonal retrograde transport pathway and their transport together with TeNT and neurotrophin receptor to the soma of motor neurons (*long-range effects*). Fast retrograde transport of these organelles requires Rab7 activity. Question marks indicate molecular events that have not been conclusively demonstrated to date and DN stands for dominant-negative mutants

Whilst it became clear that synaptic vesicle proteins play a role in the binding and uptake of BoNT, independent lines of evidence suggested that at least a fraction of these neurotoxins are internalised in an activity-independent fashion (Verderio et al. 1999; Restani et al. 2012a). These findings strongly suggest that a

proportion of BoNT is not internalised via synaptic vesicle recycling and may have additional receptors and/or additional routes of entry at nerve terminals (Fig. 2). Furthermore, BoNT/C has been shown to bind to both polysialogangliosides and phospholipids (Tsukamoto et al. 2005, 2008; Kroken et al. 2011; Strotmeier et al. 2011; Zhang and Varnum 2012), suggesting that this serotype has a unique binding modality to neuronal membranes.

The search for the protein receptor of TeNT has posed an even bigger challenge than the identification of BoNT receptors. Whilst TeNT entry into hippocampal neurons is stimulation-dependent (Matteoli et al. 1996; Blum et al. 2012), its internalisation into the NMJ and cultured motor neurons is largely independent (Schmitt et al. 1981; Deinhardt et al. 2006a) or only partially modulated (Simpson 1985) by synaptic activity. This remarkable difference suggests that TeNT may use a different endocytic mechanism to be sorted in motor neurons from other neuronal types that are blocked by this neurotoxin, such as inhibitory interneurons and hippocampal neurons (Fig. 2). This unique intracellular sorting may couple TeNT to the retrograde transport route in motor neurons in contrast to BoNT, which are preferably sorted to recycling synaptic vesicles at the NMJ. Early evidence suggested that TeNT interacts with Thy-1, a glycosylphosphatidylinositol (GPI)-anchored glycoprotein, in PC12 cells (Schiavo et al. 1991; Herreros et al. 2000). Interestingly, one or more GPI-anchored proteins are involved in TeNT binding and intracellular activity, since treatment of PC12 cells or neurons with a phosphatidylinositol-specific phospholipase C prevents the TeNT-induced cleavage of VAMP-2 (Herreros et al. 2001; Munro et al. 2001). Disruption of the integrity of membrane microdomains, which is essential for GPI-anchored protein clustering, also prevented VAMP-2 cleavage by TeNT (Herreros et al. 2001; Munro et al. 2001). However, Thy-1 is unlikely to be the only protein receptor for TeNT since mice lacking Thy-1 are only slightly less sensitive to TeNT intoxication than wild-type controls (Herreros et al. 2001). SV2 was also shown to bind TeNT in central neurons (Yeh et al. 2010). However, this latter interaction has been recently disputed (Blum et al. 2012) and no evidence is presently available validating this mechanism in motor neurons or inhibitory interneurons. Last, but not least, the possibility that the interaction of TeNT with neuronal and non-neuronal cells is mediated by the binding of polysialogangliosides to two distinct sites of its binding domain (H_CT) has been proposed (Fotinou et al. 2001; Rummel et al. 2003; Chen et al. 2008, 2009). Interestingly, immobilised glycolipid complexes have been shown to display higher affinity to H_CT (Rinaldi et al. 2009), suggesting that ganglioside *cis* interactions may have important modulatory roles in the initial binding of TeNT to the neuronal membrane.

4 Clostridial Neurotoxin Endocytosis

Once CNT are bound to their surface receptors, a complex cascade of protein–protein and protein–lipid interactions trigger the recruitment of clathrin and adaptor proteins to the inner leaflet of the plasma membrane, which marks the

onset of the endocytic process (Fig. 2). Although BoNT and TeNT are mainly internalised by distinct pathways (synaptic vesicle recycling and synaptic vesicle-independent clathrin-dependent endocytosis, respectively) (Deinhardt et al. 2006a; Montal 2010; Blum et al. 2012), several aspects of these mechanisms are shared at the molecular level. One of the early events of these processes is the recruitment of specific clathrin adaptors, which leads to the accumulation of effector proteins altering membrane curvature at endocytic sites. A major determinant of this process is the enrichment of specific lipids, such as phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P_2), at these sites. This event is likely to precede membrane curvature initiation as most of the accessory proteins, such as AP2, bind to this lipid (Haucke 2005). Several factors mediating membrane curvature possess a bin–amphiphysin–rvs (BAR) domain (Qualmann et al. 2011), which enables them to initiate and maintain membrane curvature, whilst other proteins lacking the BAR domain, such as epsins, shape membranes by inserting an amphipathic helix into the inner leaflet of the lipid bilayer, making it more accessible to clathrin (Ford et al. 2002; Hinrichsen et al. 2006). Deinhardt et al. have shown that the internalisation of TeNT relies on the canonical clathrin adaptors AP2 and AP180, but is independent of epsin1, since the overexpression of an epsin1 mutant unable to bind PtdIns(4,5)P_2 did not affect the internalisation of the toxin, whilst completely abolished transferrin uptake (Deinhardt et al. 2006a). Epsin1 was shown to target ubiquitinated receptors to the late endosomal/lysosomal pathway (Le Roy and Wrana 2005), suggesting that TeNT follows an intracellular trafficking route bypassing this sorting step. Accordingly, TeNT is known to access an atypical endosomal compartment in motor neurons, which is not acidified (Lalli et al. 2003a; Bohnert and Schiavo 2005). Thus, this epsin1-independent endocytic route may prevent the membrane insertion of HC and the subsequent translocation of the active LC into the cytoplasm, a process that is triggered by low pH, allowing the delivery of TeNT to axonal retrograde carriers in an intact form (Fig. 2). At the same time, this specific sorting avoids the targeting of TeNT to lysosomes and its degradation. As to which other membrane curvature protein(s) might act as a replacement of epsin1 remains unknown.

Following the recruitment of clathrin adaptors, a clathrin basket is formed around the pit and a new CCV is ready to be born. The last step in the biogenesis of CCV is the recruitment of a member of the dynamin family, a large GTPase, which upon GTP hydrolysis, drives the fission of the CCV from the plasma membrane. Crucially, the uptake of both TeNT and BoNT/A is disrupted in the presence of dynamin inhibitors (Deinhardt et al. 2006a; Harper et al. 2011) or by overexpression of dynamin mutants (Deinhardt et al. 2006a) (Fig. 2). Electron microscopy studies have confirmed that CNT are taken up into CCV (Black and Dolly 1986; Deinhardt et al. 2006a), which undergo uncoating in the synaptic cytoplasm.

5 Fate Decision in the Trafficking of Clostridial Neurotoxins

Although several binding and endocytic determinants are shared by BoNT and TeNT, the intracellular fate of these neurotoxins is largely distinct. In motor neurons, TeNT is internalised together with neurotrophin receptors and their ligands into transport endosomes (Fig. 2), which are characterised by near-neutral pH and low degradative potential (Bohnert and Schiavo 2005), and are delivered to the cell body. Upon arrival in the soma, TeNT is sorted to a transcytotic route and gains access to inhibitory interneurons (Schiavo et al. 2000). In contrast, BoNT are mostly taken up in synaptic vesicles and remain confined at distal synapses, such as the NMJ. During reloading with neurotransmitters, the pH drop in the lumen of synaptic vesicles determines the insertion of BoNT into the lipid bilayer (Montecucco et al. 1986, 1989) which triggers the translocation of the LC into the cytoplasm, where the disulphide bond is reduced and it can specifically cleave the synaptic targets (Koriazova and Montal 2003; Fischer and Montal 2007; Fischer et al. 2008; Montal 2010). However, recent evidence suggests that BoNT/A is also retrogradely transported in several neuronal types, including hippocampal, tectal and motor neurons (Antonucci et al. 2008; Restani et al. 2012a) and undergoes transcytosis in the visual system (Restani et al. 2011, 2012b), mimicking at least in part the behaviour of TeNT.

For its long-range transport, TeNT exploits a highly specialised trafficking pathway shaped by strong evolutionary pressure. Eukaryotic cells are characterised by a highly compartmentalised organisation and efficient communication between different cellular areas is required to ensure cellular homeostasis. Transport over long distances reaches its higher specialisation in neurons, where dendritic and axonal compartments are in dynamic equilibrium via a network of highly regulated transport routes powered by molecular motors (Hirokawa et al. 2010; Soo et al. 2011; Winckler and Yap 2011). Eukaryotic cells, including neurons, rely on three superfamilies of motor proteins: kinesins and dyneins transport their cargoes on microtubules (MT), whereas myosins are F-actin-dependent (Stiess and Bradke 2011). MT and actin microfilaments extend longitudinally within neurons, with MT mainly present in axons and dendrites and actin microfilaments enriched at synaptic regions (Hirokawa et al. 2010). Both cytoskeletal elements are characterised by a highly polarised architecture. The plus, fast-growing end of MT is directed towards the periphery in axons and distal dendrites, whereas the barbed, growing end of actin microfilaments points to the plasma membrane in pre- and post-synaptic regions. Fast axonal and dendritic transport is mainly MT-dependent and relies on kinesins and cytoplasmic dynein for the distribution within different neuronal regions of a variety of cargoes, which include organelles, proteins and RNAs (Hirokawa et al. 2010). Moreover, modulation of axonal transport by varying the concentration and/or activity of individual motors constitutes a reliable size-sensing mechanism in vitro and in vivo (Rishal et al. 2012). In axons, fast axonal transport occurs both in the anterograde (from cell body towards the periphery) and retrograde (from axonal tips to cell body) directions by means of kinesins and cytoplasmic dynein, respectively (Vale et al.

1985; Hirokawa et al. 2010). Myosins also participate in axonal transport in the proximity of the synaptic regions or in areas where the distribution of MT is less uniform (Langford 2002; Lalli et al. 2003b).

6 Axonal Transport of Tetanus Neurotoxin

Kinetic analysis of axonal transport assessed in primary motor neuron cultures using either H_CT or the directly labelled full-length neurotoxin revealed a complex speed profile, which can be deconvolved in a trimodal Gaussian distribution with peaks at 1, 1.5 and 2.1 μm/s, well within the range of fast axonal transport (Lalli and Schiavo 2002; Hafezparast et al. 2003). Two different types of pleiomorphic organelles responsible for the axonal retrograde transport of TeNT were identified: vesiculo-tubular structures, that show a continuous retrograde movement and account for the faster transport component, and round vesicles characterised by a more discontinuous and slower retrograde transport (Lalli et al. 2003a). In vivo characterisation of H_CT transport in the sciatic nerve or in sensory neurons confirmed this trimodal speed distribution (Bilsland et al. 2010) (Fig. 4). Strikingly, the average velocities observed in vivo were higher than those observed in vitro (Bilsland et al. 2010). This is likely to be due to the extensive axon myelination occurring in motor neurons in the sciatic nerve, which is known to stabilise MT, and/or differences between embryonic (in vitro) and adult (in vivo) motor neurons (Bilsland et al. 2010). In addition to a major role of kinesins, cytoplasmic dynein and MT, the retrograde transport of TeNT also relies on myosin Va, an actin-associated motor protein. Whilst the fastest component of the transport is accomplished almost totally by cytoplasmic dynein and requires kinesins at equilibrium, the intermediate component is dependent on myosin Va, as demonstrated by the transport impairments observed in myosin Va-null motor neuron cultures (Lalli et al. 2003b). The requirement of both actin microfilaments and MT for fast axonal transport is in line with the tight association occurring between these two cytoskeletal elements seen by ultrastructural analysis (Bearer and Reese 1999). Furthermore, it has been proposed that the recruitment of myosins, in addition to cytoplasmic dynein, allows neurons to assure a continuous retrograde movement of organelles also during transitions between different MT (Langford 2002; Lalli et al. 2003b).

7 Tetanus Toxin Shares Transport Compartments with Neurotrophins and Their Receptors

Many different external stimuli rely on axonal retrograde transport for reliable and fast signalling from distal synapses to the cell body. Activated receptor complexes are sorted to transport organelles, called signalling endosomes (Fig. 3), that

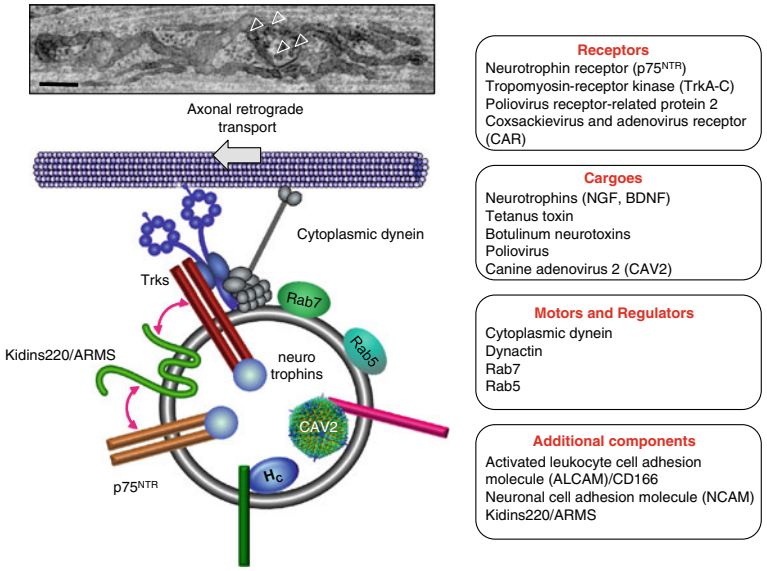

Fig. 3 Schematic representation of a TeNT-positive signalling endosome. TeNT and its binding fragment H_CT exploit the retrograde transport machinery used by physiological cargoes, such as neurotrophins (*light blue*), for their entry into the CNS. Once internalised together with its still unknown receptor(s) (*green*), H_CT enters signalling endosomes containing Trks, $p75^{NTR}$ and their interacting protein Kidins220/ARMS (kinase-D-interacting substrate of 220 kDa/ankyrin-rich membrane spanning; *green*). The progression and fast axonal transport of these signalling endosomes are dependent on the sequential recruitment of the two small GTPases Rab5 (*blue*) and Rab7 (*green*). Fast axonal retrograde transport of these signalling organelles relies on the MT-dependent motor cytoplasmic dynein (*blue*) and its associated complex dynactin (*grey*). Signalling endosomes contain several plasma membrane proteins, such as coxsackie- and adenovirus receptor (CAR, dark red), which are known to bind viruses. As such, these organelles are exploited by several viruses, such as CAV2 and poliovirus, to access the CNS. Additional components and cargoes of these axonal carriers are listed on the right. An electron microscopy image of a signalling endosome containing H_CT (empty arrows) is shown on the top. Scale bar, 200 nm

undergo fast axonal transport together with their adaptors and downstream signalling molecules, thus overcoming the limitations of signal transduction mechanisms based uniquely on diffusion (Howe 2005). Neurotrophins (NT) and their receptors, tropomyosin receptor kinase (Trk) and p75 neurotrophin receptor ($p75^{NTR}$), are among the best known examples of such retrogradely transported signalling complexes (Schecterson and Bothwell 2010). NT interact with Trks and $p75^{NTR}$ at distal sites and these activated receptor complexes are transported towards the nucleus, where they promote gene expression events controlling differentiation and survival in most types of neurons (Butowt and von Bartheld 2003; Winckler and Yap 2011). It has recently been shown that TeNT exploits the organelles used by NT receptor complexes for its journey from nerve terminals to the soma. Experiments performed in primary spinal cord motor neurons using fluorescently labelled H_CT showed that TeNT shares retrograde carriers with NGF

and $p75^{NTR}$ (Lalli and Schiavo 2002). A common route for TeNT and neurotrophin receptors was further demonstrated by showing the presence in the same transported endosome of H_CT, brain-derived neurotrophic factor (BDNF), $p75^{NTR}$ and TrkB (Deinhardt et al. 2006b). This is a general mechanism, since it has been shown both in primary motor and dorsal root ganglia (DRG) neurons. Progression towards the cell body is dependent on Rab5 and Rab7, two small GTPases with multiple roles in the endocytic pathway (Deinhardt et al. 2006b) (Fig. 3). Rab5 and Rab7 act in a sequential manner: Rab5 is involved in the initial steps of the internalisation process, whereas Rab7 is required for fast progression along axons (Deinhardt et al. 2006a; Salinas et al. 2009). This unanticipated relationship between the trafficking of TeNT and NT is further supported by independent lines of evidence. BDNF was shown to increase the efficiency and kinetic of internalisation of H_CT at the murine NMJ in a dose-dependent manner (Roux et al. 2006). Other neurotrophins, such as NT4, have similar effects on the localisation and internalisation of TeNT but are less potent than BDNF (Roux et al. 2006). The functional interaction between TeNT and NT is bidirectional, since both TeNT and H_CT activate TrkA and its downstream effectors extracellular signal-regulated kinase 1/2 (ERK1/2) and phospholipase C γ1 (PLCγ1) in a dose-dependent manner (Gil et al. 2000; Gil et al. 2001, 2003).

8 Shared Pathways with Pathogens

Neurotoxins, such as TeNT and to a lesser extent BoNT, are not the only exogenous molecules gaining access to the CNS by exploiting the axonal retrograde transport pathway. Many viruses, which are endocytosed by clathrin-dependent and -independent mechanisms, rely on controlled acidification steps for their uncoating and cytoplasmic entry (Salinas et al. 2010). Once released in the cytoplasm, these virions are transported to the nucleus by binding directly motor proteins, such as cytoplasmic dynein (Greber and Way 2006; Salinas et al. 2010). However, other neurotrophic viruses, such as canine adenovirus 2 (CAV2), are retrogradely transported together with their receptors, in TeNT-positive carriers containing $p75^{NTR}$ (Salinas et al. 2009) (Fig. 3). Poliovirus (PV) is another example of a neurotrophic virus that undergoes retrograde transport in a TeNT-positive compartment in primary motor neurons (Ohka et al. 2009). Interestingly, the kinetics of these organelles seems to be controlled by the PV receptor, probably via its ability to bind directly cytoplasmic dynein (Ohka et al. 2009). Other viruses resemble TeNT dynamics in terms of their intracellular sorting and axonal trafficking. Accordingly, the first phase of influenza virus transport is largely MT-dependent with speed ranging between 1 and 4 μm/s and a pH of its carriers approaching neutrality (Lakadamyali et al. 2003). The sequential involvement of Rab5 and Rab7 is a key feature of the sorting and retrograde transport of this virus as well as of HIV (Vidricaire and Tremblay 2005), influenza virus H3N2 (Sieczkarski and Whittaker 2003) and simian virus 40 (SV40) (Vonderheit and Helenius 2005).

Similar to TeNT, SV40 and polyoma virus bind to neuronal receptors associated with lipid rafts and are internalised by a cholesterol- and glycosphingolipid-dependent mechanism (Smith et al. 2003). Although not essential for its infectivity, $p75^{NTR}$ contributes to the binding, internalisation and axonal transport of rabies virus and its subsequent transcytosis to second-order neurons (Ugolini 1995; Tuffereau et al. 2007). In addition to viruses, amyloid beta (Aβ), prion protein (PrP) and toxic protein aggregates have been found to be associated with axonal carriers and affect axonal transport. In particular, Aβ, which has been implicated in Alzheimer's disease, was found to directly interact with $p75^{NTR}$ (Yaar et al. 1997) and to impair axonal retrograde transport of BDNF in primary neurons (Poon et al. 2011). Similarly, a toxic PrP peptide has been shown to induce cell death via direct activation of $p75^{NTR}$ signalling (Della-Bianca et al. 2001), whereas the full-length protein undergoes fast axonal transport both in peripheral and central neurons (Borchelt et al. 1994; Encalada et al. 2011). Altogether, these findings suggest that the axonal retrograde transport route is a main gateway for the entry and spread into the CNS of pathological agents and virulence factors.

9 Botulinum Neurotoxins and Their Long-Range Transport Mechanisms

BoNT activity is mainly restricted to distal synapses, a feature that is exploited in the many therapeutic applications of BoNT (Schiavo et al. 2000; Hackett and Kam 2007). In spite of the overwhelming evidence supporting this peripheral mechanism of action, several reports suggest that BoNT might also have central effects in humans and animal models. More than 50 years ago, the case of a patient affected by botulism showing clear CNS effects was reported (Polley et al. 1965). Initially, these central effects of BoNT were explained by synaptic plasticity changes occurring between the central and the peripheral neuron of the network, after the latter had been silenced by BoNT intoxication. However, the suggestion of a possible, direct, central action of BoNT arose from experiments performed by Wiegand and colleagues who detected a rise in radioactivity in the ventral horn of the spinal cord after injection of radiolabelled BoNT/A in the cat gastrocnemius muscle, both ipsilaterally and contralaterally to the site of administration (Wiegand et al. 1976). Further evidence of axonal retrograde transport of BoNT/A and BoNT/B was found in mouse muscle diaphragm after intraperitoneal administration or incubation in vitro with radiolabelled neurotoxins. Although internalisation occurred with different efficiency for BoNT/A and B, both toxins were found in the axoplasm of myelinated axons within vacuolar-like structures, thus suggesting the entry of BoNT in a specific endocytic pathway (Black and Dolly 1986). Direct evidence supporting the hypothesis that BoNT/A is retrogradely transported in an active form and undergoes transcytosis in second-order neurons was provided by the detection of BoNT/A-cleaved SNAP-25 in cholinergic synapses in rat retina

after injection of the neurotoxin in the superior colliculus (SC) (Antonucci et al. 2008). No BoNT/A-cleaved SNAP-25 was detected in synapses of the retina when this neurotoxin was injected in SC after MT depolymerisation, thus ruling out diffusion-based spreading mechanisms. Supporting evidence for a long-range action was also obtained by detecting changes in the activity of CA1 pyramidal neurons after injection of BoNT/A into the contralateral hippocampus (Antonucci et al. 2008).

Interestingly, transport (and transcytosis) of catalytically active BoNT/A occurs not only in the retrograde direction, but also anterogradely. This unexpected property of BoNT/A has been demonstrated by detecting the presence of neurotoxin-cleaved SNAP-25 in the SC after BoNT/A injection in the contralateral retina (Restani et al. 2011).

Interestingly, different BoNT serotypes display distinct long-range effects in vivo. Conversely to BoNT/A, BoNT/E seems unable to alter the activity of CA1 neurons after injection in the contralateral hippocampus (Antonucci et al. 2008), in spite of overwhelming evidence demonstrating its silencing activity upon ipsilateral injection (Costantin et al. 2005). This result suggests distinct sorting pathways and/or distal kinetics for different BoNT serotypes (Antonucci et al. 2008; Caleo and Schiavo 2009). This conclusion seems to be supported by overt differences in the rate of SNAP-25 cleavage induced by BoNT/A and BoNT/E in sympathetic neurons cultured in Campenot chambers (Lawrence et al. 2012). After distal application of these neurotoxins, BoNT/A-cleaved SNAP-25 was readily detectable in cell bodies, whilst the BoNT/E truncated form of this synaptic protein was only slightly detectable in the somas, despite a much higher quantity of BoNT/E being used in this experiment (Lawrence et al. 2012). The documented short-lived enzymatic activity of BoNT/E was suggested to be responsible for this lack of detection (Lawrence et al. 2012).

However, direct evidence supporting a differential rate of transport of BoNT/A and BoNT/E is still lacking. Experiments recently performed in our laboratory filled this gap by analysing the kinetic properties of the axonal transport of these serotypes once internalised in primary spinal cord motor neurons (Restani et al. 2012a). Full-length BoNT/A and BoNT/E, or their atoxic binding fragments undergo axonal retrograde transport in non-acidic organelles with speed profiles matching fast MT-dependent transport and largely overlapping with TeNT-positive carriers in living motor neurons (Restani et al. 2012a). Our analysis suggests that a lower transport efficiency of BoNT/E compared to BoNT/A may contribute to the differential in vivo effects of these two serotypes (Caleo and Schiavo 2009). Whilst BoNT/A undergoes fast and continuous retrograde transport, BoNT/E-positive organelles show a discontinuous movement towards the cell body, characterised by a higher frequency of pauses and short periods of anterograde transport (Restani et al. 2012a). Altogether, these results demonstrate that BoNT undergo axonal retrograde transport in neurons both in vitro and in vivo.

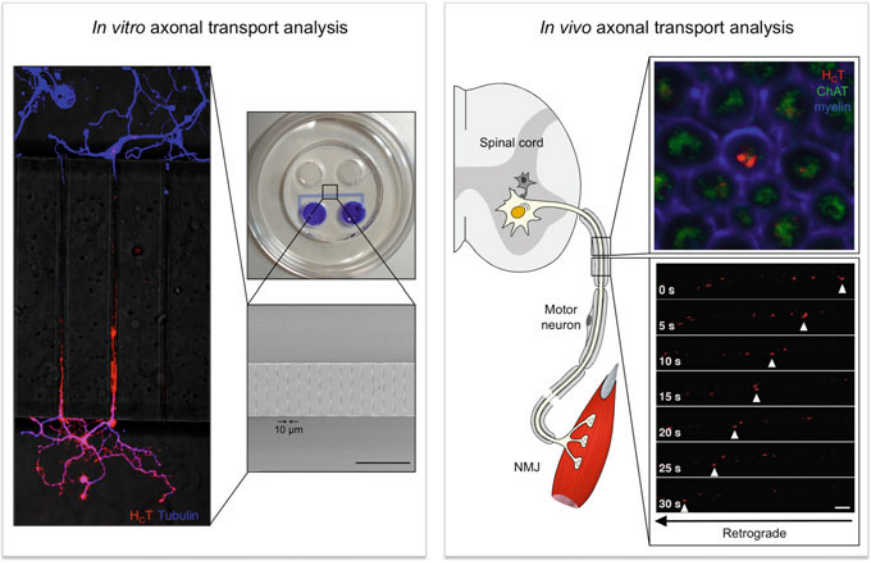

Fig. 4 Innovative strategies to monitor axonal transport in vitro and in vivo. *Left panel*. Microfluidic chambers allow the physical separation of distal axons and synapses (*bottom*) from cell bodies (*top*) in a variety of neuronal cultures, including motor neurons. HcT (*red*), added only to the axonal side, is transported towards the cell bodies located in the top compartment. The two compartments are separated by small microgrooves (10 µm width), which allow the passage of axons, but not of cell bodies. These compartments are also microfluidically separated, a feature that impedes the passive diffusion of ligands and drugs between the two sides of the device. Motor neurons are stained for *β*-tubulin (*blue*). Scale bar, 500 µm. *Right panel*. Axonal transport of HcT can be monitored in the intact sciatic nerve. After intramuscular injection in the mouse hindlimb, fluorescent HcT (*red*) was found within axons of the sciatic nerve (*top*), which have been stained for choline acetyl transferase (ChAT, *green*), a motor neuron marker, and myelin (*blue*). This technique allows a quantitative kinetic analysis of axonal transport of HcT in vivo (Bilsland et al. 2010). Still images from a confocal movie show an H_CT-positive endosome undergoing fast axonal retrograde transport (*arrowheads*). The cell body is out of view on the left. Scale bar, 5 µm

10 Future Perspectives

In spite of the wealth of data presently available, there are important aspects of the interaction of BoNT with the neuronal membrane that deserve further investigations. Among these the role of different isoforms of SV2 and Syt in the binding of BoNT to distinct neuronal subtypes is presently unclear. Similarly, very little is known about the role of specific posttranslational modifications on the affinity of SV2 and Syt to BoNT and on their intracellular trafficking. It is also unclear whether phospholipids and polysialogangliosides are sufficient to mediate high affinity binding and internalisation of BoNT/C and other serotypes in neurons.

Strikingly, the protein receptor(s) of TeNT at the NMJ is still unknown, although SV2 seems to play a role in TeNT binding to interneurons (Yeh et al. 2010). Previous data suggest that GPI-anchored proteins play a role in TeNT intoxication (Herreros et al. 2001; Munro et al. 2001), but it is unclear whether their contribution is direct or mediated through changes in the clustering of polysialogangliosides on the synaptic plasma membrane. Future experiments are necessary to elucidate the nature of this high affinity TeNT receptor complex and its putative role in axonal retrograde transport and intracellular sorting.

The quantitative analysis of the retrograde transport of different BoNT is still in its infancy. To date, little is known about the molecular mechanisms controlling this process, its modulators and additional cargoes sharing the same transport organelles. As significant differences between the axonal transport of BoNT/A and BoNT/E have recently been detected (Lawrence et al. 2012; Restani et al. 2012a), the CNT field is now facing the exciting prospect of better understanding the molecular determinants of this differential neuronal trafficking, and the relationship between the 3D structure of these serotypes and their intracellular fate. Furthermore, additional studies are necessary to assess whether other serotypes might be transported as well. Novel in vitro approaches, such as compartmentalized motor neuron cultures in microfluidic devices (Fig. 4) will be instrumental for the analysis of the axonal transport of these neurotoxins and the definition of the cellular machinery controlling their trafficking.

Last but not least, the molecular mechanism of transcytosis of TeNT from motor neurons into interneurons is still poorly characterised, as well as the pathway followed by BoNT/A for its transneuronal migration in the visual system (Restani et al. 2011, 2012b).

These studies will not only enable a better characterisation of the CNT at molecular level, but they will also shed light on fundamental processes essential for neuronal homeostasis and survival, providing new potential strategies for the delivery of therapeutics to the CNS.

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