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Abstract: Tetanus (TeNT) and botulinum (BoNT) neurotoxins, the causative agents of tetanus and botulism, respectively, are the most potent toxic molecules known to mankind. This extreme potency is attributed to: i) their specificity for essential components of the neurotransmitter release machinery present at vertebrate synapses, and ii) their high-affinity targeting to motor neurons by binding to polysialogangliosides and protein receptors. Comprising the clostridial neurotoxin family, TeNT and BoNTs engage distinct surface receptors and intracellular sorting pathways in neurons. BoNTs bind to the intraluminal domain of specific synaptic vesicle proteins that are exposed to the extracellular milieu upon exocytosis, and are taken up by synaptic vesicle recycling. A sizeable proportion of BoNT molecules remain at the neuromuscular junction, where their protease moiety is released into the cytoplasm, blocking synaptic transmission and causing flaccid paralysis. In contrast, TeNT undergoes binding to specific components of the basal membrane at the neuromuscular junction, is endocytosed into motor neurons and sorted to axonal signalling endosomes. Following this, TeNT is transported to the soma of motor neurons located in the spinal cord or brainstem, and then transcytosed to inhibitory interneurons, where it blocks synaptic transmission. TeNT-induced impairment of inhibitory input leads to hyperactivity of motor neurons, causing spastic paralysis, which is the hallmark of tetanus. This review examines the molecular mechanisms leading to the entry, sorting and intracellular trafficking of TeNT and BoNTs.
The travel diaries of tetanus and botulinum neurotoxins

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Abstract

Tetanus (TeNT) and botulinum (BoNT) neurotoxins, the causative agents of tetanus and botulism, respectively, are the most potent toxic molecules known to mankind. This extreme potency is attributed to: i) their specificity for essential components of the neurotransmitter release machinery present at vertebrate synapses, and ii) their high-affinity targeting to motor neurons by binding to polysialogangliosides and protein receptors. Comprising the clostridial neurotoxin family, TeNT and BoNTs engage distinct surface receptors and intracellular sorting pathways in neurons. BoNTs bind to the intraluminal domain of specific synaptic vesicle proteins that are exposed to the extracellular milieu upon exocytosis, and are taken up by synaptic vesicle recycling. A sizeable proportion of BoNT molecules remain at the neuromuscular junction, where their protease moiety is released into the cytoplasm, blocking synaptic transmission and causing flaccid paralysis. In contrast, TeNT undergoes binding to specific components of the basal membrane at the neuromuscular junction, is endocytosed into motor neurons and sorted to axonal signalling endosomes. Following this, TeNT is transported to the soma of motor neurons located in the spinal cord or brainstem, and then transcytosed to inhibitory interneurons, where it blocks synaptic transmission. TeNT-induced impairment of inhibitory input leads to hyperactivity of motor neurons, causing spastic paralysis, which is the hallmark of tetanus. This review examines the molecular mechanisms leading to the entry, sorting and intracellular trafficking of TeNT and BoNTs.

Highlights

- Tetanus and botulinum neurotoxins undergo long range traffic in mammalian neurons
- Signalling endosomes and autophagomes mediate the transport of these neurotoxins
- The binding of tetanus toxin to the basal membrane is key for its uptake in neurons

1. Historical background

Tetanus (TeNT) and botulinum (BoNT) neurotoxins have been studied intensely over the last century, while BoNTs have attracted worldwide attention in the last 25 years for their ever-increasing medical applications. These neurotoxins are produced by Clostridium tetani and various serotypes of Clostridium botulinum, which together form the clostridial neurotoxin (CNT) family. The unmistakable clinical symptoms of tetanus toxicity were first reported in Egyptian and Indian documents before 1500 BC. It was Hippocrates (460–370 BC) who coined the term τετανοσ (translated to ‘tension’ in Ancient Greek) to describe these symptoms when studying the progressive spastic paralysis developed by a sailor as a consequence of an injury caused while handling the anchor of his boat (Udwadia, 1994).
However, the aetiology of tetanus remained a mystery until the end of the 19th century, when the efforts of Carle and Rattone in Turin, Nicolaier in Göttingen and Kitasato in Berlin led to the conclusion that tetanus was a transmissible disease caused by an anaerobic sporigenic bacterium present in the soil (Udwadia, 1994). Although Nicolaier was able to report the presence of a strychnine-like substance in the supernatant of these bacterial cultures, it was Faber in 1890 who isolated TeNT and demonstrated its physiological role as the causative agent of the spastic paralysis observed during tetanus (Udwadia, 1994). Importantly, the availability of methods to isolate TeNT subsequently allowed Marie in 1897, and Meyer, Ranson and others thereafter to demonstrate that TeNT was able to reach the central nervous system (CNS), mediating its central affects after travelling along peripheral motor nerves (Habermann, 1989; Marie, 1897; Udwadia, 1994). These findings, thus, set the stage for the modern analyses of CNT trafficking in neurons.

Botulism, characterised by a general muscle weakness, was described independently in the same period as TeNT by Kerner (1822), followed by the isolation of C. botulinum and the first serotype of BoNT by van Ermengem in 1895 (van Ermengem, 1979). Traditionally seven BoNT serotypes have been described in the literature including BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F, and BoNT/G (Montal, 2010; Poulain et al., 2015; Pirazzini et al., 2017). However, most recently, an eighth BoNT serotype has been discovered and named BoNT/X (Zhang et al., 2017). Each individual serotype contains multiple subtypes of toxins (e.g., BoNT/A1, BoNT/A2, etc.) (Poulain et al., 2015) with unique activities, synaptic targets and downstream intracellular signalling (Pirazzini et al., 2017).

These discoveries, together with the isolation of different C. botulinum toxigenic strains and studies on their intracellular activity and synaptic targets of TeNT and BoNTs in the 1990s (Montal, 2010; Pirazzini et al., 2017), have revealed important insights into a complex protein machinery responsible for the neuronal targeting, uptake and inhibition of synaptic transmission by these neurotoxins. As a consequence, the study of the mode of action of TeNT and BoNTs continue to have direct impact on several disciplines, including microbiology, pharmacology, physiology, cell biology, biochemistry and molecular medicine. TeNT and BoNTs have been used as tools of discovery in bioscience to dissect the mechanisms of regulated secretion and intracellular trafficking, and as CNS-targeting molecules for DNA vaccines and therapeutics (Behzadi et al., 2016; Toivonen et al., 2010).

The need for further in-depth characterisation of the mechanism of action of these neurotoxins both in vitro and in vivo is further highlighted by the widespread use of BoNTs to treat pathologies beyond the classical area of synaptic hyperactivity, such as chronic migraine, depression and aesthetic/dermatological applications (Pirazzini et al., 2017). In contrast, tetanus continues to claim the lives of thousands of individuals per year, including
many newborns affected by *tetanus neonatorum* [1](http://apps.who.int/gho/data/view.main.1520_46) making the development of efficient countermeasures an urgent priority.

2. Mechanism of Action

TeNT displays lethal dose, 50% (LD$_{50}$) ranging between 0.1 and 5 ng/kg of body weight in mice (Gill, 1982), while the BoNT LD$_{50}$ lies between 0.1 and 500 ng/kg (Pirazzini et al., 2017). The LD$_{50}$ for both neurotoxins, however, can greatly vary in different species (Gill, 1982). There are many factors that determine the precise time of symptom onset (i.e., paralysis) after CNT intoxication, including dose, route of application and species. For TeNT, the incubation period between the initial injury and the onset of clinical symptoms is highly variable (from 1-2 days to a couple of months) (Udwadia, 1994) and includes the time needed for the spores to germinate into vegetative bacteria, which, after autolysis, presumably release the neurotoxin into the bloodstream. Shorter incubation periods are usually associated with TeNT of higher severity, in which the symptoms reach their peak in 7-10 days, plateau for 1-2 weeks and gradually decline in additional 1-2 weeks, although muscle stiffness may persists for weeks or even months after recovery (Udwadia, 1994). BoNTs, on the other hand, are typically released into the body via food contaminated by spores, in which the storage conditions allowed their germination and the expression of the progenitor toxin complex formed by BoNTs and non-toxic neurotoxin-associated proteins (NAPs). NAPs comprise a non-toxic non-haemagglutinin component (NTNH$_A$) that plays an important role in protecting BoNTs from the harsh gastrointestinal tract, and other subunits that enable binding to the surface of intestinal cells for subsequent transcytosis of the neurotoxic complex from the apical membrane to the basolateral membrane of intestinal epithelium (Amatsu et al., 2013; Gu et al., 2012; Lee et al., 2013; Lee et al., 2014; Sugawara et al., 2014; Yao et al., 2014). Once released, the BoNT progenitor complex sequesters E-cadherin in its monomeric form, blocking E-cadherin dimer formation, thus weakening the trans-epithelial barrier (Lee et al., 2014; Sugawara et al., 2014). This process leads to bulk entry of neurotoxin into the bloodstream and can accelerate intoxication.

After entering the general circulation, TeNT and BoNTs bind with high affinity to the presynaptic membrane of the motor neuron at the neuromuscular junction (NMJ) where they are rapidly internalised (Montal, 2010; Rummel, 2016) (Figure 1A). BoNTs mainly remain at the NMJ and inhibit the release of the excitatory neurotransmitter acetylcholine (Figure 1), blocking muscle excitation-contraction coupling and thus causing a flaccid paralysis. In contrast, TeNT enters motor neuron axon terminals through endocytosis at the NMJ (Figure...
and is predominantly retrogradely transported in axonal signalling endosomes to the soma of motor neurons in the spinal cord (Schmieg et al., 2014) (Figure 1C). TeNT is subsequently transcytosed into inhibitory interneurons where it blocks neuroexocytosis through the cleavage of the SNARE VAMP/synaptobrevin, thus inhibiting neurotransmitter release from intoxicated interneurons to motor neurons (Figure 1D). As a consequence, the balance between excitatory and inhibitory inputs to motor neurons is disrupted, eliciting hyperactive motor neurons and spasticity. In addition to inhibitory interneurons (i.e., glycinergic and GABAergic), excitatory interneurons (i.e., glutamatergic and cholinergic) also respond to TeNT application but with different sensitivity and effects (Bergey et al. 1987; McMahon et al., 1992; Williamson et al. 1992; Shin et al., 2012). This preference for inhibitory versus excitatory synapses is maintained when TeNT is applied directly into the CNS and underlie the neurodegenerative and epileptogenic effects of TeNT (Bagetta et al., 1990; Bowery et al., 1992; Ferecsko et al., 2015), which may result from unopposed release of glutamate from excitatory central synapses.

Paradoxically, despite TeNT and BoNTs exert opposing influences on skeletal muscle (i.e., spasticity versus flaccidity), their modes of action are quite similar. Indeed, both CNT family members block neurotransmitter release via specific cleavage of soluble NSF-attachment protein receptor (SNARE) proteins involved in neuroexocytosis (Montecucco et al., 2005). The differences in clinical symptoms arise from preferential site of action in different neurons (Montal, 2010; Rummel, 2016) (Figure 1).

Interestingly, the hallmarks of TeNT and BoNT have also been observed in neurons other than motor neurons, including cortical, sensory and sympathetic neurons (Blum et al., 2014; Cordero-Erausquin et al., 2009).

3. Multi-domain structure and function

TeNT and BoNTs are remarkably similar in sequence and structure (Montal, 2010). The 150 kDa single-chain proteins are cleaved by proteases producing an active neurotoxin comprising two chains of 100 kDa (heavy or H chain) and 50 kDa (light or L chain), which remain associated via non-covalent interactions and a conserved inter-chain disulphide bond essential for neurotoxicity (de Paiva et al., 1993; Pirazzini et al., 2014; Schiavo et al., 1990). The heavy chain is further subdivided into two 50 kDa domains: the amino terminal (H_N) and carboxy terminal (H_C) domains (Montal, 2010). X-ray crystallography of BoNT/A (Garcia-Rodriguez et al., 2007; Lacy et al., 1998; Stevens et al., 1991), BoNT/B (Swaminathan and Eswaramoorthy, 2000), BoNT/E (Kumaran et al., 2009) and TeNT (Masuyer et al., 2017) was used to confirm the spatial orientation of these domains relative to each other. TeNT and
BoNT/E assume a more compact/closed arrangement, with the Hc domain interacting closely with the L chain and Hn, although distinct interaction surfaces are employed by the two CNTs (Kumaran et al., 2009; Masuyer et al., 2017). Conversely, BoNT/A and BoNT/B display an elongated arrangement of the three domains, which are largely separated, with the exception of an extended loop in the amino-terminus of the H chain (termed belt), which is wrapped around the L chain.

The Hc domain of CNTs is responsible for their neuron-specific binding and is composed of two sub-domains of roughly the same size (Pirazzini et al., 2017). While the amino-terminal sub-domain (Hcn) is structurally similar to the carbohydrate-binding domain of the lectin family, the carboxy-terminal sub-domain (Hcc) is homologous to domains involved in protein-protein interactions (Montal, 2010; Pirazzini et al., 2017). It is in the Hcc loops of CNTs where the highest degree of sequence and structural divergence lies (Lacy and Stevens, 1999), which ultimately contributes to binding specificity. Crucially, in BoNT/A and BoNT/E, the Hc domain is isolated from the remaining part of the molecule, allowing full access of all surface loops for binding. The close conformation found in BoNT/E and TeNT may instead impose some steric constraints to the full accessibility of Hc to protein and lipid receptors (Kumaran et al., 2009; Masuyer et al., 2017). Moreover, other portions of TeNT may contribute to enhanced clearance from the NMJ and wider spreading into spinal cord neurons (Ovsepian et al., 2015).

The Hc domains of CNTs bind to polysialogangliosides on the plasma membrane, in particular to G1b gangliosides, with high specificity and affinity (Montecucco, 1986), although binding to other gangliosides series has been reported (e.g. BoNT/A interacts with GQ1b and GT1b, but also to GD1a, albeit with lower affinity) (Kitamura et al., 1980; Takamizawa et al., 1986). Binding to polysialogangliosides is facilitated by oligosaccharide-binding sites (one and two, in BoNTs and TeNT, respectively) in the Hcc sub-domain of the heavy chain (Rummel, 2016; Rummel et al., 2003). Mutations in the carbohydrate binding domain abrogate binding of these toxins to neuronal plasma membranes, thus highlighting the importance of this interaction (Rummel, 2016). Addition of the polysialoganglioside GT1b to NMJs protects the neuron from the toxic effects of BoNT via competitive inhibition and partially abolishes the retrograde transport of TeNT (Stoeckel et al., 1977). In addition, removal of sialic acid residues from the plasma membrane by neuraminidase treatment (Bigalke et al., 1986) or blocking ganglioside biosynthesis (Kitamura et al., 2005; Rummel, 2013; Williamson et al., 1999) inhibits CNT activity. Despite the strong requirement of surface polygangliosides for uptake of CNTs, it is clear that they are not unique determinants of binding since TeNT and BoNTs do not compete with each other for internalisation at the NMJ. Additional protein receptor(s) have therefore been suggested to act in conjunction with
gangliosides, referred to as the dual receptor hypothesis (Montecucco, 1986; Rummel, 2016; Rummel et al., 2007). According to this hypothesis, polysialogangliosides act in one of two ways: i) recruit TeNT and BoNTs to specific regions of the plasma membrane, which are locally enriched in a certain protein receptor, or ii) maintain a specific conformational state of these toxins so as to enable the receptor to bind. In line with this hypothesis, specific protein co-receptors have been identified for most CNTs (see section 4).

The pH-dependent translocation of the L-chain from the endocytic lumen into the cytosol is mediated by the amino-terminal part of the H chain (HN). HN is composed of a belt closely interacting with the L chain and a central portion containing two very long α-helices (Montal, 2010; Pirazzini et al., 2016). Although the function of this domain in membrane insertion was first described in the 1980s, the exact mechanism underlying the transfer of the L chain to the cytosol remains, at least in part, controversial (Montal, 2010; Pirazzini et al., 2016). Recent findings have demonstrated that the reduction of the disulphide bridge linking the H and L chains by the thioredoxin reductase-thioredoxin (TrxR-Trx) system is required for the release of the L chain into the cytosol, and inhibition of TrxR-Trx activity prevents the intoxication of neurons both in vitro and in vivo (Pirazzini et al., 2015; Pirazzini et al., 2014; Zanetti et al., 2015). Reduction of the interchain disulphide bridge is strictly coupled to L chain refolding, since the inhibition of cytosolic chaperone Hsp90 reduces the intracellular activity of BoNTs (Azarnia Tehran et al., 2017). Interestingly, Hsp90 and TrxR-Trx physically interact on the surface of SVs, where they orchestrate a chaperone-redox complex likely to be involved in synaptic protein refolding, which is exploited by the L chains of CNTs to enter the cytosol (Azarnia Tehran et al., 2017).

The L chain contains the catalytic zinc atom and is responsible for the intracellular endopeptidase activity of CNTs, which is directed towards the SNARE proteins VAMP/synaptobrevin 1-3 (BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/HA and BoNT/X, TeNT), SNAP25 (BoNT/A, BoNT/C and BoNT/E) and syntaxin-1 (Montal, 2010; Pirazzini et al., 2016; Zhang et al., 2017). BoNT/X also cleaves the non-canonical substrates VAMP4, VAMP5 and Ykt6 (Zhang et al., 2017). The number of zinc atoms that bind to the L chain varies among different CNTs; while the L chains of TeNT, BoNT/A, BoNT/B and BoNT/F chelate one atom of zinc (Schiavo et al., 1992a; Schiavo et al., 1992b; Schiavo et al., 1993), BoNT/C binds two atoms of zinc with different affinities (Breidenbach and Brunger, 2005; Garcia-Rodriguez et al., 2007; Schiavo et al., 1995). The protease activity of the L chain can be abolished by heavy metal chelators, such as ortho-phenantroline, thus generating inactive apo-neurotoxins (Bhattacharyya and Sugiyama, 1989; Schiavo et al., 1992a). The zinc atom is chelated by two histidines located in the endopeptidase motif (His-Glu-x-x-His); the glutamic acid residue in this motif binds the water molecule necessary for the catalysis (third
ligand), with another glutamic acid (Glu261 in BoNT/A) acts as the fourth ligand (Montal, 2010; Pirazzini et al., 2016).

4. Neuron-specific binding

CNTs are exquisitely neuron-specific and able to bind neurons in vivo at concentrations in the sub-nanomolar regime (Simpson, 2000). Both BoNTs and TeNT interact with the peripheral cholinergic nerve terminals, while TeNT also binds to sympathetic and adrenergic nerve fibres (Rossetto et al., 2001). The H\textsubscript{C} domains are largely responsible for this high-affinity binding, since paralysis caused by native toxins can be counteracted by recombinant H\textsubscript{C} proteins (Lalli et al., 1999; Rummel et al., 2009). Additional targeting information, however, may be encoded elsewhere in the full length neurotoxin (Ovsepian et al., 2015). The neuron-specificity of CNTs is also likely to reflect the complexity of their cellular receptors, which are most probably composed of multiple lipid and protein components forming arrays of presynaptic receptors (APRs) (Montecucco et al., 2004). Polysialogangliosides play a key role in the binding and internalisation of CNTs at the presynaptic membrane, presumably due to their high concentration at the NMJ and their lateral mobility. Interaction of the toxin with polysialogangliosides allows its subsequent interaction with other molecules in the APRs, thus leading to virtually irreversible binding. In addition to polysialogangliosides, APRs contain lipids such as cholesterol and sphingomyelin, GPI-anchored protein(s) and other membrane-bound protein(s) (Montecucco et al., 2004). Interestingly, both TeNT and BoNT/A have been found to bind sphingomyelin-enriched membrane microdomains (Herreros et al., 2001; Muraro et al., 2009); additionally, BoNT/A and BoNT/C interact with phosphoinositol lipids (Muraro et al., 2009; Tsukamoto et al., 2005; Zhang and Varnum, 2012). Since BoNTs and TeNT have been proposed to bind distinct co-receptors, the APRs recognised by BoNTs would direct them inside vesicles that are acidified within the NMJ, such as recycling synaptic vesicles (SVs), whereas the APRs binding to TeNT would sort this neurotoxin into signalling endosomes undergoing axonal retrograde transport towards the neuronal soma (Schmieg et al., 2014).

Several lines of evidence indicate that BoNTs enter the NMJ by exploiting the process of SV recycling (Montal, 2010; Pirazzini et al., 2017). Accordingly, many CNTs bind to the intraluminal domain of SV proteins, which are exposed to the extracellular milieu upon SV exocytosis (Figure 1B). BoNT/B, BoNT/D, BoNT/C and BoNT/G interact with the calcium-sensing proteins synaptotagmin-1 and/or -2 (reviewed in Rummel, 2016). Multiple isoforms of the synaptic vesicle protein-2 (SV2) function as the protein receptors for BoNT/A, BoNT/E and BoNT/F (Rummel, 2016), whilst BoNT/C and BoNT/D seem to utilize only gangliosides
as host cell receptors (Karalewitz et al., 2012). Due to its recent discovery, no protein receptor has been described for BoNT/X (Zhang et al., 2017). Crucially, Harper et al. found that BoNT/A is internalised in a SV subpopulation that is not destined for recycling, highlighting the existence of functional heterogeneity between SV pools (Harper et al., 2016).

BoNT/A, similar to TeNT, is able to enter neurons when SV recycling is blocked (Restani et al., 2012a), suggesting that BoNT/A could potentially use alternative entry route(s) targeting this neurotoxin to sites other than the NMJ (Figure 1B). In agreement, BoNT/A has been shown to be retrogradely transported in hippocampal, tectal and motor neurons and undergo transcytosis in the visual system (Bomba-Warczak et al., 2016; Mazzocchio and Caleo, 2015). Additionally, BoNT/A accumulates in dorsal root ganglia upon injection in the bladder (Papagiannopoulou et al., 2016). Although SV2A can potentially undergo long-range transport in spinal cord motor neurons (Debaisieux et al., 2016), other protein receptors may be involved in this process. One such protein whose endogenous trafficking route might be exploited by BoNT/A is the fibroblast growth factor receptor-3 (FGFR3). Although controversial (Weisemann et al., 2016), FGFR3 has been shown to bind BoNT/A (Jacky et al., 2013). FGFR3 undergoes receptor-mediated endocytosis (Haugsten et al., 2011) and has been identified in the proteome of axonal signalling endosomes (Debaisieux et al., 2016), thus suggesting an alternative transport route for BoNT/A. On the other hand, BoNT/A might bind to the basal membrane at the NMJ, as recently reported for TeNT (Bercsenyi et al., 2014), leading to its sorting to axonal signalling endosomes and transcytosis.

To reach its final site of action, TeNT must enter two different types of neurons: a motor neuron innervating skeletal muscle followed by an inhibitory interneuron of the spinal cord (Figure 1A,B,D). Post-internalisation, TeNT is sorted to different intracellular pathways, hence it is expected to bind to distinct receptors in these neurons. Several lines of evidence indicate that TeNT and BoNTs are internalised via different routes. First, TeNT at physiological concentrations does not block synaptic transmission at the NMJ, unlike BoNTs. Second, if TeNT binding sites were present in recycling SVs, then an increase in the rate of neuronal stimulation should lead to increased binding of the toxin to the membrane. This, however, is not observed. While high frequency stimulation increases the rate of TeNT intoxication, it does not enhance binding of the toxin to the NMJ (Schmitt et al., 1981). Third, the abrogation of exocytosis and neurotransmitter release from NMJs by BoNT treatment does not affect the uptake and retrograde axonal transport of TeNT (Habermann and Erdmann, 1978). Fourth, TeNT exhibits temperature-sensitive binding and internalisation; while fully functional at 25°C, it is inactive on NMJs at 18°C even in the presence of high-frequency stimulation and massive neurotransmitter release (Schmitt et al., 1981).
Due to the presence of two ganglioside-binding sites in the H$_C$ domain of TeNT, it was proposed to rely solely on lipid binding for its cellular entry (Chen et al., 2009). Cis-interactions of gangliosides have been suggested to play an important role in mediating binding of the neurotoxin to target cells (Rinaldi et al., 2009). However, since polysialogangliosides are not uniquely distributed at the NMJ and are not readily internalised (Deinhardt et al., 2006a), TeNT would require additional factors to enter into motor neurons. One of the proteins described to interact with TeNT is Thy-1, an abundant GPI-anchored protein (Herreros et al., 2001). However, Thy-1 is unlikely to be the main protein receptor on motor neurons in vivo because mice lacking Thy-1 remain sensitive to the toxic effects of TeNT (Herreros et al., 2001). TeNT enters motor neurons together with the neurotrophin receptors TrkB and p75NTR (Deinhardt et al., 2006b; Terenzio et al., 2014a; Terenzio et al., 2014b), and its internalisation is dependent on neurotrophin signalling. Interestingly, TeNT interacts with specific basal membrane components at the NMJ to stimulate uptake of TrkB and formation of signalling endosomes (Bercsenyi et al., 2014). In particular, the H$_C$ domain of TeNT (H$_C$T) directly binds to nidogen-1 and -2 (also known as entactin-1 and -2) and selectively targets NMJs rich in nidogen-2. A small peptide derived from nidogen-1 blocks TeNT uptake in motor neurons and at NMJs, and protects mice from TeNT-induced paralysis (Bercsenyi et al., 2014). Nidogen-2 knockout mice are less sensitive to tetanus intoxication and show TeNT-mediated botulism-like symptoms (Bercsenyi et al., 2014), which are also observed when TeNT is injected in wild type animals at high doses (Matsuda et al., 1982). Taken together, these results suggest that TeNT and BoNTs might share common entry routes when key basal membrane components required by TeNT are absent or when its preferred internalisation pathway is overloaded. Accordingly, addition of recombinant nidogen-1 decreases the co-localisation of H$_C$T with SV2A and increases its rate of internalisation, whilst at high concentrations, H$_C$T preferentially enters SV2A-positive organelles (Bercsenyi et al., 2014). Although controversial (Blum et al., 2012), TeNT was also shown to bind SV2 in hippocampal neurons and relied on this interaction for cell entry (Yeh et al., 2010).

The identification of protein co-receptors for TeNT at the NMJ provides crucial information on this trafficking pathway from the NMJ to spinal cord interneurons, offering new strategies for the delivery of therapeutics into the spinal cord. Furthermore, it provides new insights into the alternative trafficking pathway used by BoNT/A to elicit responses in the CNS (Caleo and Schiavo, 2009). Although further studies are required to determine whether BoNTs engage with basal membrane components, these findings open the possibility that extracellular matrix-derived peptides might be used to mitigate some of the undesired long-range effects of BoNT/A therapy in humans.
5. Neuronal internalisation and axonal transport

Endocytosis of CNTs is an active process: their cellular entry is temperature- and energy-dependent and is differentially modulated by synaptic activity (Baldwin and Barbieri, 2007; Blum et al., 2014; Pirazzini et al., 2017; Rummel et al., 2009). At physiological concentrations, uptake of both CNTs occurs via distinct mechanisms with TeNT internalisation predominantly occurring through clathrin-mediated endocytosis whilst BoNTs exploit SV recycling (Blum et al., 2012; Deinhardt et al., 2006a; Montal, 2010). TeNT internalisation is dependent on a specific subset of clathrin adaptors, which target the neurotoxin to non-acidified endosomal compartments (Bohnert and Schiavo, 2005), thus preventing the translocation of the L chain into the cytoplasm of the motor neuron and enabling its arrival in a fully active form to spinal cord inhibitory interneurons. Internalisation of BoNT/A and TeNT is partially abrogated by dynamin inhibitors (Deinhardt et al., 2006a; Harper et al., 2011) or dynamin mutant overexpression (Deinhardt et al., 2006a), in agreement with the established role of dynamins in the fission of clathrin-coated vesicles from the plasma membrane.

Although TeNT and its atoxic H chain fragment (H_C_T) uptake in motor neurons is largely unaffected by membrane depolarisation (Deinhardt et al., 2006a), their mechanism of entry in central neurons is likely to be dependent on SV recycling. Experiments by Blum et al. indicate that H_C_T entry in cortical neurons is stimulated by membrane depolarisation (Blum et al., 2014), validating previous results that show TeNT internalisation in hippocampal neurons follows SV re-uptake (Matteoli et al., 1996). However, subtle differences may exist between H_C_T and TeNT uptake and trafficking in cortical and spinal cord neurons, as recently reported (Blum et al., 2014).

Post-internalisation, TeNT must undergo long-range transport to reach the soma of motor neurons, from where it undergoes trans-synaptic transfer into inhibitory interneurons. In order to achieve this, it exploits endogenous microtubule-based axonal transport pathways which the neuron uses to communicate between the synapse and the soma (Goldstein and Yang, 2000) (Figure 1C). This highly regulated, long-range axonal transport is facilitated by two classes of microtubule-dependent molecular motors: cytoplasmic dynein and kinesins. Cytoplasmic dynein motor proteins are responsible for moving cargo in the retrograde direction from axonal terminals to the cell body, where the minus ends of microtubules are located. In contrast, kinesin motor proteins are responsible for delivering their cargo in the anterograde direction toward the plus end of microtubules that are located in synaptic terminals or growth cones (Hirokawa et al., 2010; Vale, 2003). Despite the majority of
transport dynamics involving microtubules (Hirokawa et al., 2010), actin-based motors (e.g., myosins) also contribute and hence, some form of interactions between the microtubule- and actin-mediated transport systems has been suggested (Hirokawa et al., 2010; Vale, 2003). Cytoplasmic dynein plays a particularly crucial role in the retrograde transport of TeNT to the soma (Lalli et al., 2003; Schiavo et al., 2013) (Figure 1C). In vivo studies using mice carrying a mutation in cytoplasmic dynein heavy chain showed deficits in axonal retrograde transport of HcT, which are associated with motor and sensory neuron degeneration (Hafezparast et al., 2003). Functional axonal transport is crucial for the development and maintenance of the nervous system, and impairments in this process are associated with neurodegenerative conditions, such as amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease and acquired peripheral neuropathies (De Vos and Hafezparast, 2017; Schiavo et al., 2013). However, for cargo to bind, dynein must form a complex with dynactin and this formation is dependent on the Bicaudal D (BICD) family of adaptor proteins that are enriched at the minus-end of microtubules (Carter et al., 2016; Hoogenraad and Akhmanova, 2016). Underpinning their importance, BICD1 is involved in the trafficking of TeNT and neurotrophin-receptor complexes (Schmieg et al., 2014; Terenzio et al., 2014b) and mutations in the homologous BICD2 have been shown to cause spinal muscular atrophy (Oates et al., 2013; Rossor et al., 2015).

The retrograde transport of HcT takes place in axonal signalling endosomes, which contain neurotrophins, their receptors and other proteins (Deinhardt et al., 2006b; Lalli and Schiavo, 2002). To create a functional physical map of these organelles, our laboratory has developed a method based on magnetic iron oxide nanoparticles coupled to HcT, which enable the purification of signalling endosomes from embryonic stem cell-derived motor neurons and their quantitative mass spectrometry analysis (Debaisieux et al., 2016; Deinhardt et al., 2006b; Wade et al., 2012). We found that HcT-positive organelles undergo rapid maturation with the acquisition of late endosomal markers, and are specifically enriched in proteins known to be involved in neurodegenerative diseases and neuroinfection (Debaisieux et al., 2016). The maturation of signalling endosomes is dependent upon Rab5, which is involved in sorting after internalisation, followed by Rab7, which is involved in the fast retrograde transport of HcT (Figure 1C) as well as neurotrophin-receptor complexes (Deinhardt et al., 2006b; Salinas et al., 2009). A functional cross-talk between HcT and neurotrophins is emerging, since the application of exogenous brain-derived neurotrophic factor (BDNF) results in an increase in the internalisation of HcT at the NMJ as well as accumulation of HcT in the sciatic nerve (Roux et al., 2006). However, the sharing of axonal signalling endosomes by other virulence/pathological factors such as canine adenovirus-2, cholera toxin, poliovirus, Borna virus and pseudotyped lentivirus with neurotrophin receptors suggests that despite

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different methods of internalisation, a common mechanism for sorting and retrograde transport may exist (Charlier et al., 2016; Hislop et al., 2014; Ohka et al., 2009; Salinas et al., 2009).

In contrast to the acidic pH found in the lumen of the majority of endosomes, axonal transport carriers containing HcT display neutral pH. The pH of signalling endosomes is critical, as acidification in TeNT and BoNTs carriers triggers the translocation of the enzymatically active subunit (i.e., L chain) into the cytosol. Endosomal acidification also causes the dissociation of neurotrophin-receptor complexes, terminates their en route signalling and targets the endosome for degradation. Such tight regulation of the pH is dependent on the vacuolar ATPase complex (Bohnert and Schiavo, 2005). Therefore, the maintenance of neutral pH of TeNT carriers enables its presentation to interneurons in a fully active form to consequently mediate the disruption of synaptic communication.

In contrast to the historical view that BoNTs only disrupt communication at the NMJ, several studies provide evidence of long-range trafficking and CNS expression of BoNT after intramuscular injections (reviewed in Caleo and Schiavo, 2009; Mazzocchio and Caleo, 2015). Indeed, BoNT/A was first detected in the spinal cord ventral horn after injections in the gastrocnemius muscle (Wiegand et al., 1976). Its presence was also detected in diaphragms after intraperitoneal injections of BoNT/A and /B (Black and Dolly, 1986). In this context, higher BoNT/A levels were observed in the axoplasm of myelinated axons, suggestive of differences in the uptake and sorting mechanisms of different BoNT serotypes (Black and Dolly, 1986). Experiments comparing the effects of BoNT/A and /E applied to the distal neurites of primary sympathetic neurons cultured in compartmentalised chambers revealed that whilst most BoNT/A and /E cleaved SNAP25 near the sight of uptake, a small fraction also cleaved SNAP25 in their soma, albeit at different rates (Lawrence et al., 2012). In addition, Restani et al. have demonstrated that BoNT/A undergoes fast axonal retrograde transport whereas BoNT/E exhibited slower axonal retrograde transport with a greater frequency of pausing and short periods of anterograde transport in primary motor neurons (Restani et al., 2012a). This study suggests that BoNT/E is coupled with a less efficient mechanism of long-range trafficking and may explain, in part, why BoNT/E cannot mediate similar effects in the CNS, despite having the same intracellular targets as BoNT/A.

Furthermore, these data also suggest that the serotype and concentration of BoNTs are also key factors in local (i.e., NMJ) versus distant (i.e., soma) effects. These results were confirmed in hippocampal neurons grown in microfluidic devices, where BoNT/A and BoNT/D were found to be taken up into non-acidified organelles undergoing axonal retrograde transport to the soma (Bomba-Warczak et al., 2016). After internalisation, their activities were detected in upstream neurons, thus indicating that BoNT/A, BoNT/D and TeNT may undergo...
interneuronal transfer in an active form *in vitro* (Bomba-Warczak et al., 2016). Interestingly, Wang *et al.* found that a significant proportion of H₃ fragment of BoNT/A (H₃A) was incorporated into LC3-positive autophagosomes in hippocampal neurons, which then underwent retrograde transport to the cell soma. Blocking autophagosome formation or acidification inhibited the activity-dependent retrograde trafficking of H₃A, suggesting a role for presynaptic autophagosomes in long distance transport of BoNT/A (Wang *et al.*, 2015).

Elements of this process have been recapitulated *in vivo* by studies demonstrating the retrograde transport of BoNT/A and H₃A in spinal cord motor neurons (Antonucci *et al.*, 2008; Restani *et al.*, 2012a; Restani *et al.*, 2012b; Wang *et al.*, 2015) and sensory neurons (Fan *et al.*, 2017; Hong *et al.*, 2017; Matak *et al.*, 2014; Papagiannopoulou *et al.*, 2016).

Taken altogether, these investigations provide evidence that BoNTs also undergo retrograde transport to the CNS, the consequences of which are yet to be entirely understood.

### 6.0 Future perspectives

Since TeNT and BoNTs are capable of being sorted to the axonal retrograde trafficking route and undergo interneuronal transfer *in vivo*, it has been proposed that non-toxic fragments of CNTs may be used as targeting agents for the delivery of therapeutics, such as recombinant proteins and/or DNA, into the CNS (Toivonen *et al.*, 2010). Chimeras of H$_2$T and various proteins have been shown to be successfully internalised and undergo axonal retrograde transport, maintaining their enzymatic activity upon delivery to the targeted area (Francis *et al.*, 2004a). Importantly, these H$_2$T fusion proteins were shown to transfer across synapses *in vivo* (Coen *et al.*, 1997), access second and higher-order neurons (Miana-Mena *et al.*, 2003) and deliver their payload to the neuronal cytosol, when fused to translocation-competent proteins (e.g. diphtheria toxin) (Francis *et al.*, 2004b).

Due to their diverse biological activities, neuronal growth factors have frequently been used as biological payloads. BDNF and glial cell line-derived neurotrophic factor (GDNF) fused with H$_2$T have been found to have neuroprotective effects in animal models of ALS (Calvo *et al.*, 2011; Ciriza *et al.*, 2008) and Parkinson’s disease (Larsen *et al.*, 2006). Fusion of cardiotrophin-1 and H$_2$T also promoted motor neuron survival (Bordet *et al.*, 2001), whilst a chimera of the anti-apoptotic factor Bcl-XL and H$_2$T decreased apoptosis induced by glutamate-mediated excitotoxicity (Carlton *et al.*, 2008). BDNF has also been targeted to neurons by nanoparticles made of polyethylene imine linked to H$_2$T (Oliveira *et al.*, 2010).

Protein engineering has been explored to re-target BoNTs to different neuronal populations by using a self-assembling ‘protein stapling’ technology (Ferrari *et al.*, 2013). BoNT/A lacking its H$_3$A domain as well as H$_2$T were produced separately and then linked by exploiting the
high-affinity interaction of paired SNARE motifs (Ferrari et al., 2013). The stapled chimera was found to lack peripheral paralytic effects, and significantly reduce the enhanced nociceptive sensitivity found in animal models of inflammatory, surgical, and neuropathic pain (Mangione et al., 2016).

Whilst these studies have explored the potential of recombinant protein chimeras, a few attempts have been made to directly express these fusion proteins by delivering exogenous DNA. In particular, Moreno-Igoa et al. showed that a single intramuscular administration of naked-DNA encoding GDNF-HcT significantly delayed the onset of symptoms, ameliorate the functional deficits and extended the lifespan of a mouse model of ALS (Moreno-Igoa et al., 2012). HcT might thus represent a valuable strategy to deliver therapeutics to the CNS by exploiting its high tropism for motor neurons and its ability to undergo axonal retrograde transport and transcytosis. In addition, DNA fusion vaccines encoding a portion of HcT coupled with tumour antigen sequences is highly immunogenic against colon carcinoma (Behzadi et al., 2016).

Several studies have also highlighted the intrinsic ability of HcT to protect neurons from neurodegeneration in a variety of animal models, including chemically induced Parkinson’s disease (Mendieta et al., 2009), ALS (Moreno-Igoa et al., 2010) and spinal muscular atrophy (Olivan et al., 2016). This property may be linked to the ability of HcT to activate the neurotrophin receptor signalling cascade, including ERK1/2 and Akt, via a mechanism still not completely understood (Gil et al., 2003; Gil et al., 2001). HcT co-localises with the neurotrophin receptors TrkB and p75NTR in axonal signalling endosomes (Deinhardt et al., 2006a; Lalli and Schiavo, 2002), yet it is unclear whether HcT signalling is physiologically relevant and whether it would negatively or positively regulate axonal retrograde transport. However, recent results from Wang et al. demonstrate that TrkB activation couples synaptic activity with the retrograde flux of axonal signalling endosomes, thus suggesting that HcT and TeNT regulate their own sorting and/or retrograde transport (Wang et al., 2016).

In addition to their importance as virulence factors and biotherapeutics, BoNTs, TeNT and their recombinant fragments are also becoming increasingly popular as key tools of discovery to uncover deficits of axonal transport in animal models of neurological diseases (Bilsland et al., 2010; LeRoux et al., 2014; Malik et al., 2011; Schafer et al., 2017; Sleigh et al., 2017a; Sleigh et al., 2017b), ageing (Sleigh and Schiavo, 2016) and as flexible trans-synaptic tracers (Coen et al., 1999; Kumar and Boehm, 2014).

Several important questions centred on the trafficking of BoNTs and TeNT are still unaddressed. First and foremost, the nature of the receptor complex targeting TeNT and BoNTs to axonal signalling endosomes at the NMJ need to be elucidated at the molecular
level, together with the exact role of neurotrophin signalling (or other signalling cascades) in this process. This line of research would help the identification of the minimal requirements for the efficient sorting of these neurotoxins to proximal and/or distal sites of action. This information would be important for basic and clinical scientists to direct the in vivo activity of BoNTs, thus improving their clinical specificity and limiting their side effects. Further research is also necessary to define the neuronal receptors of the expanding family of BoNT subtypes (Peck et al., 2017) and their preferential site of action in vivo. This in turn would allow the selection of novel BoNT subtypes endowed with unique pharmacodynamics and pharmacokinetics properties ideal for specific clinical applications (e.g. chronic pain, short term treatment in post-operative management). In this way, the travel diaries of TeNT and BoNTs would become not just a fascinating reading for molecular and cellular neurobiologists, but a very useful roadmap for pharmacologists and clinical neuroscientists to understand, navigate and treat the human nervous system.

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Figure legend

**Figure 1. Trafficking of the tetanus (TeNT) and botulinum neurotoxins (BoNT) in vivo** (adapted from Schmeig et al., 2014b). A. Anatomical connections between skeletal muscles, spinal cord motor neurons and their afferent cells. Motor neurons innervate skeletal muscles via the neuromuscular junction (NMJ). The motor neuron axon is myelinated and can reach over a meter in length in humans. The motor neuron soma is located in the spinal cord, where it forms contacts with adjacent interneurons and upper motor neurons. **B. Internalisation at the NMJ.** Both TeNT (T; in blue) and BoNTs (B; in green) accumulate in the synaptic space of the NMJ, which is filled with basal lamina (in yellow). TeNT binds to polysialogangliosides and nidogens, and this complex is targeted to the axonal retrograde transport route (solid blue arrow). At higher doses or with the unavailability of nidogens, TeNT is able to bind SV2 and can enter synaptic vesicle (SV) recycling at the NMJ (thinner blue arrow) (Bercsenyi et al., 2014). The majority of BoNT molecules remain at the NMJ (solid green arrow), where they cleave synaptic SNAREs, thereby blocking the fusion of (SVs) containing acetylcholine and causing flaccid paralysis. However, a fraction of BoNT/A may enter organelles targeted to the soma (thinner green arrow), such as axonal signalling endosomes (Restani et al., 2012a) or autophagosomes (Wang et al., 2015). **C. Axonal retrograde transport.** TeNT is transported to the soma via axonal signalling endosomes, along with neurotrophins and their receptors. This long-range retrograde axonal transport, which also requires the GTP-bound form of the small GTPase Rab7 (in purple) (Deinhardt et al., 2006b), is dependent on the microtubule-based motor, cytoplasmic dynein (in red). **D. Interneuronal transfer of TeNT into inhibitory interneurons.** Once in the motor neuron soma in the spinal cord (Bilsland et al., 2010), TeNT is released into the extracellular medium and is internalised by SV recycling into inhibitory interneurons, where it cleaves VAMP/synaptobrevin, thereby blocking inhibitory neurotransmission. This impairs the balance between inhibitory and excitatory afferents on the motor neurons, leading to disruptions in co-ordinated muscle contraction and spastic paralysis.