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Corresponding Author: Professor Giampietro Schiavo,

Corresponding Author's Institution: University College London

First Author: Sunaina Surana

Order of Authors: Sunaina Surana; Andrew P Tosolini; Ione F Meyer; Alexander D Fellows; Sergey S Novoselov; Giampietro Schiavo

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

Sunaina Surana[1,*], Andrew P. Tosolini[1,*], Ione F. G. Meyer[1,2], Alexander D. Fellows[1], Sergey S. Novoselov[1] and Giampietro Schiavo[1,3,4‡]

[1]Sobell Department of Motor Neuroscience & Movement Disorders, UCL Institute of Neurology, University College London, London WC1N 3BG, UK

[2]MRC Centre for Neuromuscular Diseases, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, UK

[3]Discoveries Centre for Regenerative and Precision Medicine, University College London Campus, London WC1N 3BG, UK

[4]UK Dementia Research Institute at UCL, London WC1E 6BT, UK

*These authors have contributed equally to this work

[‡]Corresponding author: Professor Giampietro Schiavo FMedSci FRSB, Sobell Department of Motor Neuroscience & Movement Disorders, UCL Institute of Neurology, University College London, London WC1N 3BG, UK. Phone: 0044 7918 738393. e-mail: giampietro.schiavo@ucl.ac.uk

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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* (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 (NTNHA) 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**

1B) 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 H_C domain interacting closely with the L chain and H_N, 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 H_C 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 (H_{CN}) is structurally similar to the carbohydrate-binding domain of the lectin family, the carboxy-terminal sub-domain (H_{CC}) is homologous to domains involved in protein-protein interactions (Montal, 2010; Pirazzini et al., 2017). It is in the H_{CC} 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 H_C 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 H_C 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 H_C 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 H_{CC} 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 (H_N). H_N 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_C domains are largely responsible for this high-affinity binding, since paralysis caused by native toxins can be counteracted by recombinant H_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 $p75^{NTR}$ (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_CT) 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_CT with SV2A and increases its rate of internalisation, whilst at high concentrations, H_CT 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_CT) 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_CT 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_CT 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 H_CT, 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 H_CT 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 H_CT, 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 H_CT-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 H_CT 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

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 H_CT 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 H_C fragment of BoNT/A (H_CA) 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 H_CA, 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 H_CA 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_CT 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_CT 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_CT 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_CT also promoted motor neuron survival (Bordet et al., 2001), whilst a chimera of the anti-apoptotic factor Bcl-XL and H_CT 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_CT (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_CA domain as well as H_CT 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-H_CT 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). H_CT 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 H_CT coupled with tumour antigen sequences is highly immunogenic against colon carcinoma (Behzadi et al., 2016).

Several studies have also highlighted the intrinsic ability of H_CT 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 H_CT 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). H_CT co-localises with the neurotrophin receptors TrkB and $p75^{NTR}$ in axonal signalling endosomes (Deinhardt et al., 2006a; Lalli and Schiavo, 2002), yet it is unclear whether H_CT 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 H_CT 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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8. References

Amatsu, S., Sugawara, Y., Matsumura, T., Kitadokoro, K., Fujinaga, Y., 2013. Crystal structure of Clostridium botulinum whole hemagglutinin reveals a huge triskelion-shaped molecular complex. J. Biol. Chem. 288, 35617-35625.

Antonucci, F., Rossi, C., Gianfranceschi, L., Rossetto, O., Caleo, M., 2008. Long-distance retrograde effects of botulinum neurotoxin A. J. Neurosci. 28, 3689-3696.

Azarnia Tehran, D., Pirazzini, M., Leka, O., Mattarei, A., Lista, F., Binz, T., Rossetto, O., Montecucco, C., 2017. Hsp90 is involved in the entry of clostridial neurotoxins into the cytosol of nerve terminals. Cell. Microbiol. in press

Bagetta, G., Knott, C., Nistico, G., Bowery, N.G., 1990. Tetanus toxin produces neuronal loss and a reduction in GABAA but not GABAB binding sites in rat hippocampus. Neurosci. Lett. 109, 7-12.

Baldwin, M.R., Barbieri, J.T., 2007. Association of botulinum neurotoxin serotypes a and B with synaptic vesicle protein complexes. Biochemistry 46, 3200-3210.

Behzadi, E., Halabian, R., Hosseini, H.M., Fooladi, A.A., 2016. Bacterial toxin's DNA vaccine serves as a strategy for the treatment of cancer, infectious and autoimmune diseases. Microb. Pathog. 100, 184-194.

Bercsenyi, K.*, et al.*, 2014. Tetanus toxin entry. Nidogens are therapeutic targets for the prevention of tetanus. Science 346, 1118-1123.

Bhattacharyya, S.D., Sugiyama, H., 1989. Inactivation of botulinum and tetanus toxins by chelators. Infect. Immun. 57, 3053-3057.

Bigalke, H., Muller, H., Dreyer, F., 1986. Botulinum A neurotoxin unlike tetanus toxin acts via a neuraminidase sensitive structure. Toxicon 24, 1065-1074.

Bilsland, L.G., Sahai, E., Kelly, G., Golding, M., Greensmith, L., Schiavo, G., 2010. Deficits in axonal transport precede ALS symptoms in vivo. Proc. Natl. Acad. Sci. U. S. A. 107, 20523-20528.

Black, J.D., Dolly, J.O., 1986. Interaction of 125I-labeled botulinum neurotoxins with nerve terminals. I. Ultrastructural autoradiographic localization and quantitation of distinct membrane acceptors for types A and B on motor nerves. J. Cell Biol. 103, 521-534.

Blum, F.C., Chen, C., Kroken, A.R., Barbieri, J.T., 2012. Tetanus Toxin and Botulinum Toxin A Utilize Unique Mechanisms To Enter Neurons of the Central Nervous System. Infect. Immun. 80, 1662-1669.

Blum, F.C., Tepp, W.H., Johnson, E.A., Barbieri, J.T., 2014. Multiple domains of tetanus toxin direct entry into primary neurons. Traffic 15, 1057-1065.

Bohnert, S., Schiavo, G., 2005. Tetanus toxin is transported in a novel neuronal compartment characterized by a specialized pH regulation. J. Biol. Chem. 280, 42336-42344.

584 Bomba-Warczak, E., Vevea, J.D., Brittain, J.M., Figueroa-Bernier, A., Tepp, W.H., Johnson,
585 E.A., Yeh, F.L., Chapman, E.R., 2016. Interneuronal Transfer and Distal Action of Tetanus
586 Toxin and Botulinum Neurotoxins A and D in Central Neurons. Cell Rep 16, 1974-1987.

587 Bordet, T., Castelnau-Ptakhine, L., Fauchereau, F., Friocourt, G., Kahn, A., Haase, G., 2001.
588 Neuronal targeting of cardiotrophin-1 by coupling with tetanus toxin C fragment. Mol. Cell.
589 Neurosci. 17, 842-854.

590 Bowery, N.G., Bagetta, G., Nistico, G., Britton, P., Whitton, P., 1992. Intrahippocampal
591 tetanus toxin produces generalized convulsions and neurodegeneration in rats: antagonism
592 by NMDA receptor blockers. Epilepsy Res. Suppl. 9, 249-256.

593 Breidenbach, M.A., Brunger, A.T., 2005. 2.3 A crystal structure of tetanus neurotoxin light
594 chain. Biochemistry 44, 7450-7457.

595 Caleo, M., Schiavo, G., 2009. Central effects of tetanus and botulinum neurotoxins. Toxicon
596 54, 593-599.

597 Calvo, A.C*., et al.*, 2011. Lack of a synergistic effect of a non-viral ALS gene therapy based
598 on BDNF and a TTC fusion molecule. Orphanet J. Rare Dis. 6, 10.

599 Carlton, E., Teng, Q., Federici, T., Yang, J., Riley, J., Boulis, N.M., 2008. Fusion of the
600 tetanus toxin C fragment binding domain and Bcl-xL for protection of peripheral nerve
601 neurons. Neurosurgery 63, 1175-1184.

602 Charlier, C.M., Debaisieux, S., Foret, C., Thouard, A., Schiavo, G., Gonzalez-Dunia, D.,
603 Malnou, C.E., 2016. Neuronal retrograde transport of Borna disease virus occurs in signalling
604 endosomes. J. Gen. Virol. 97, 3215-3224.

605 Chen, C., Fu, Z.J., Kim, J.J.P., Barbieri, J.T., Baldwin, M.R., 2009. Gangliosides as High
606 Affinity Receptors for Tetanus Neurotoxin. J. Biol. Chem. 284, 26569-26577.

607 Ciriza, J*., et al.*, 2008. A genetic fusion GDNF-C fragment of tetanus toxin prolongs survival
608 in a symptomatic mouse ALS model. Restor. Neurol. Neurosci. 26, 459-465.

609 Coen, L., Kissa, K., le Mevel, S., Brulet, P., Demeneix, B.A., 1999. A somatic gene transfer
610 approach using recombinant fusion proteins to map muscle-motoneuron projections in
611 Xenopus spinal cord. Int. J. Dev. Biol. 43, 823-830.

612 Coen, L., Osta, R., Maury, M., Brulet, P., 1997. Construction of hybrid proteins that migrate
613 retrogradely and transynaptically into the central nervous system. Proc. Natl. Acad. Sci. U. S.
614 A. 94, 9400-9405.

615 Cordero-Erausquin, M., Allard, S., Dolique, T., Bachand, K., Ribeiro-da-Silva, A., De
616 Koninck, Y., 2009. Dorsal horn neurons presynaptic to lamina I spinoparabrachial neurons
617 revealed by transynaptic labeling. J. Comp. Neurol. 517, 601-615.

618 de Paiva, A., Poulain, B., Lawrence, G.W., Shone, C.C., Tauc, L., Dolly, J.O., 1993. A role
619 for the interchain disulfide or its participating thiols in the internalization of botulinum

neurotoxin A revealed by a toxin derivative that binds to ecto-acceptors and inhibits transmitter release intracellularly. J. Biol. Chem. 268, 20838-20844.

De Vos, K.J., Hafezparast, M., 2017. Neurobiology of axonal transport defects in motor neuron diseases: Opportunities for translational research? Neurobiol. Dis., DOI: 10.1016/j.nbd.2017.1002.1004.

Debaisieux, S., Encheva, V., Chakravarty, P., Snijders, A.P., Schiavo, G., 2016. Analysis of Signaling Endosome Composition and Dynamics Using SILAC in Embryonic Stem Cell-Derived Neurons. Mol. Cell. Proteomics 15, 542-557.

Deinhardt, K., Berninghausen, O., Willison, H.J., Hopkins, C.R., Schiavo, G., 2006a. Tetanus toxin is internalized by a sequential clathrin-dependent mechanism initiated within lipid microdomains and independent of epsin 1. J. Cell Biol. 174, 459-471.

Deinhardt, K., Salinas, S., Verastegui, C., Watson, R., Worth, D., Hanrahan, S., Bucci, C., Schiavo, G., 2006b. Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. Neuron 52, 293-305.

Fan, C., Chu, X., Wang, L., Shi, H., Li, T., 2017. Botulinum toxin type A reduces TRPV1 expression in the dorsal root ganglion in rats with adjuvant-arthritis pain. Toxicon 133, 116-122.

Ferecsko, A.S., Jiruska, P., Foss, L., Powell, A.D., Chang, W.C., Sik, A., Jefferys, J.G., 2015. Structural and functional substrates of tetanus toxin in an animal model of temporal lobe epilepsy. Brain Struct Funct 220, 1013-1029.

Ferrari, E*., et al.*, 2013. Synthetic self-assembling clostridial chimera for modulation of sensory functions. Bioconjug. Chem. 24, 1750-1759.

Francis, J.W., Bastia, E., Matthews, C.C., Parks, D.A., Schwarzschild, M.A., Brown, R.H., Jr., Fishman, P.S., 2004a. Tetanus toxin fragment C as a vector to enhance delivery of proteins to the CNS. Brain Res. 1011, 7-13.

Francis, J.W*., et al.*, 2004b. A survival motor neuron:tetanus toxin fragment C fusion protein for the targeted delivery of SMN protein to neurons. Brain Res. 995, 84-96.

Garcia-Rodriguez, C., Levy, R., Arndt, J.W., Forsyth, C.M., Razai, A., Lou, J., Geren, I., Stevens, R.C., Marks, J.D., 2007. Molecular evolution of antibody cross-reactivity for two subtypes of type A botulinum neurotoxin. Nat. Biotechnol. 25, 107-116.

Gil, C., Chaib-Oukadour, I., Aguilera, J., 2003. C-terminal fragment of tetanus toxin heavy chain activates Akt and MEK/ERK signalling pathways in a Trk receptor-dependent manner in cultured cortical neurons. Biochem. J. 373, 613-620.

Gil, C., Chaib-Oukadour, I., Blasi, J., Aguilera, J., 2001. HC fragment (C-terminal portion of the heavy chain) of tetanus toxin activates protein kinase C isoforms and phosphoproteins involved in signal transduction. Biochem. J. 356, 97-103.

656 Gill, D.M., 1982. Bacterial toxins: a table of lethal amounts. Microbiol. Rev. 46, 86-94.

657 Goldstein, L.S., Yang, Z., 2000. Microtubule-based transport systems in neurons: the roles of
658 kinesins and dyneins. Annu. Rev. Neurosci. 23, 39-71.

659 Gu, S., Rumpel, S., Zhou, J., Strotmeier, J., Bigalke, H., Perry, K., Shoemaker, C.B.,
660 Rummel, A., Jin, R., 2012. Botulinum neurotoxin is shielded by NTNHA in an interlocked
661 complex. Science 335, 977-981.

662 Habermann, E., 1989. Clostridial neurotoxins and the central nervous system: sunctional
663 studies on isolated preparations, in: Simpson, L.L. (Ed.), Botulinum neurotoxins and tetanus
664 toxin. Academic Press, San Diego.

665 Habermann, E., Erdmann, G., 1978. Pharmacokinetic and histoautoradiographic evidence for
666 the intraaxonal movement of toxin in the pathogenesis of tetanus. Toxicon 16, 611-623.

667 Hafezparast, M.*, et al.*, 2003. Mutations in dynein link motor neuron degeneration to defects
668 in retrograde transport. Science 300, 808-812.

669 Harper, C.B.*, et al.*, 2011. Dynamin Inhibition Blocks Botulinum Neurotoxin Type A
670 Endocytosis in Neurons and Delays Botulism. J. Biol. Chem. 286, 35966-35976.

671 Harper, C.B.*, et al.*, 2016. Botulinum neurotoxin type-A enters a non-recycling pool of
672 synaptic vesicles. Sci. Rep. 6, 19654.

673 Haugsten, E.M., Zakrzewska, M., Brech, A., Pust, S., Olsnes, S., Sandvig, K., Wesche, J.,
674 2011. Clathrin- and dynamin-independent endocytosis of FGFR3-implications for signalling.
675 PLoS One 6, e21708.

676 Herreros, J., Ng, T., Schiavo, G., 2001. Lipid rafts act as specialized domains for tetanus
677 toxin binding and internalization into neurons. 12, 2947-2960.

678 Hirokawa, N., Niwa, S., Tanaka, Y., 2010. Molecular motors in neurons: transport
679 mechanisms and roles in brain function, development, and disease. Neuron 68, 610-638.

680 Hislop, J.N., Islam, T.A., Eleftheriadou, I., Carpentier, D.C., Trabalza, A., Parkinson, M.,
681 Schiavo, G., Mazarakis, N.D., 2014. Rabies virus envelope glycoprotein targets lentiviral
682 vectors to the axonal retrograde pathway in motor neurons. J. Biol. Chem. 289, 16148-
683 16163.

684 Hong, B., Yao, L., Ni, L., Wang, L., Hu, X., 2017. Antinociceptive effect of botulinum toxin A
685 involves alterations in AMPA receptor expression and glutamate release in spinal dorsal horn
686 neurons. Neuroscience 357, 197-207.

687 Jacky, B.P.*, et al.*, 2013. Identification of fibroblast growth factor receptor 3 (FGFR3) as a
688 protein receptor for botulinum neurotoxin serotype A (BoNT/A). PLoS Pathog. 9, e1003369.

689 Karalewitz, A.P., Fu, Z., Baldwin, M.R., Kim, J.J., Barbieri, J.T., 2012. Botulinum neurotoxin
690 serotype C associates with dual ganglioside receptors to facilitate cell entry. J. Biol. Chem.
691 287, 40806-40816.

692 Kitamura, M., Igimi, S., Furukawa, K., Furukawa, K., 2005. Different response of the
693 knockout mice lacking b-series gangliosides against botulinum and tetanus toxins. Biochim.
694 Biophys. Acta 1741, 1-3.

695 Kitamura, M., Iwamori, M., Nagai, Y., 1980. Interaction between Clostridium botulinum
696 neurotoxin and gangliosides. Biochim. Biophys. Acta 628, 328-335.

697 Kumar, D., Boehm, U., 2014. Conditional genetic transsynaptic tracing in the embryonic
698 mouse brain. J Vis Exp. DOI: 10.3791/52487.

699 Kumaran, D., Eswaramoorthy, S., Furey, W., Navaza, J., Sax, M., Swaminathan, S., 2009.
700 Domain organization in Clostridium botulinum neurotoxin type E is unique: its implication in
701 faster translocation. J. Mol. Biol. 386, 233-245.

702 Lacy, D.B., Stevens, R.C., 1999. Sequence homology and structural analysis of the
703 clostridial neurotoxins. J. Mol. Biol. 291, 1091-1104.

704 Lacy, D.B., Tepp, W., Cohen, A.C., DasGupta, B.R., Stevens, R.C., 1998. Crystal structure
705 of botulinum neurotoxin type A and implications for toxicity. Nat. Struct. Biol. 5, 898-902.

706 Lalli, G., Bohnert, S., Deinhardt, K., Verastegui, C., Schiavo, G., 2003. The journey of
707 tetanus and botulinum neurotoxins in neurons. Trends Microbiol. 11, 431-437.

708 Lalli, G., Herreros, J., Osborne, S.L., Montecucco, C., Rossetto, O., Schiavo, G., 1999.
709 Functional characterisation of tetanus and botulinum neurotoxins binding domains. J. Cell
710 Sci. 112 2715-2724.

711 Lalli, G., Schiavo, G., 2002. Analysis of retrograde transport in motor neurons reveals
712 common endocytic carriers for tetanus toxin and neurotrophin receptor $p75^{NTR}$. J. Cell Biol.
713 156, 233-239.

714 Larsen, K.E.*, et al.*, 2006. A glial cell line-derived neurotrophic factor (GDNF):tetanus toxin
715 fragment C protein conjugate improves delivery of GDNF to spinal cord motor neurons in
716 mice. Brain Res. 1120, 1-12.

717 Lawrence, G.W., Ovsepian, S.V., Wang, J., Aoki, K.R., Dolly, J.O., 2012. Extravesicular
718 intraneuronal migration of internalized botulinum neurotoxins without detectable inhibition of
719 distal neurotransmission. Biochem. J. 441, 443-452.

720 Lee, K.*, et al.*, 2013. Structure of a bimodular botulinum neurotoxin complex provides insights
721 into its oral toxicity. PLoS Pathog. 9, e1003690.

722 Lee, K., Zhong, X., Gu, S., Kruel, A.M., Dorner, M.B., Perry, K., Rummel, A., Dong, M., Jin,
723 R., 2014. Molecular basis for disruption of E-cadherin adhesion by botulinum neurotoxin A
724 complex. Science 344, 1405-1410.

725 LeRoux, L.G., Bredow, S., Grosshans, D., Schellingerhout, D., 2014. Molecular imaging
726 detects impairment in the retrograde axonal transport mechanism after radiation-induced
727 spinal cord injury. Mol. Imaging Biol. 16, 504-510.

728 Malik, B., Nirmalananthan, N., Bilsland, L.G., La Spada, A.R., Hanna, M.G., Schiavo, G.,
729 Gallo, J.M., Greensmith, L., 2011. Absence of disturbed axonal transport in spinal and bulbar
730 muscular atrophy. Hum. Mol. Genet. 20, 1776-1786.

731 Mangione, A.S., Obara, I., Maiaru, M., Geranton, S.M., Tassorelli, C., Ferrari, E., Leese, C.,
732 Davletov, B., Hunt, S.P., 2016. Nonparalytic botulinum molecules for the control of pain. Pain
733 157, 1045-1055.

734 Marie, A., 1897. Recherche sur la toxine tetanique. Ann. Inst. Past. 11, 591-599.

735 Masuyer, G., Conrad, J., Stenmark, P., 2017. The structure of the tetanus toxin reveals pH-
736 mediated domain dynamics. EMBO Rep 18, 1306-1317.

737 Matak, I., Rossetto, O., Lackovic, Z., 2014. Botulinum toxin type A selectivity for certain types
738 of pain is associated with capsaicin-sensitive neurons. Pain 155, 1516-1526.

739 Matsuda, M., Sugimoto, N., Ozutsumi, K., Hirai, T., 1982. Acute Botulinum-Like Intoxication
740 by Tetanus Neurotoxin in Mice. Biochem Bioph Res Comm 104, 799-805.

741 Matteoli, M., Verderio, C., Rossetto, O., Iezzi, N., Coco, S., Schiavo, G., Montecucco, C.,
742 1996. Synaptic vesicle endocytosis mediates the entry of tetanus neurotoxin into
743 hippocampal neurons. Proc. Natl. Acad. Sci. U. S. A. 93, 13310-13315.

744 Mazzocchio, R., Caleo, M., 2015. More than at the neuromuscular synapse: actions of
745 botulinum neurotoxin A in the central nervous system. Neuroscientist 21, 44-61.

746 Mendieta, L., Venegas, B., Moreno, N., Patricio, A., Martinez, I., Aguilera, J., Limon, I.D.,
747 2009. The carboxyl-terminal domain of the heavy chain of tetanus toxin prevents
748 dopaminergic degeneration and improves motor behavior in rats with striatal MPP(+)-lesions.
749 Neurosci. Res. 65, 98-106.

750 Miana-Mena, F.J., Munoz, M.J., Ciriza, J., Soria, J., Brulet, P., Zaragoza, P., Osta, R., 2003.
751 Fragment C tetanus toxin: a putative activity-dependent neuroanatomical tracer. Acta
752 Neurobiol. Exp. (Wars.) 63, 211-218.

753 Montal, M., 2010. Botulinum Neurotoxin: A Marvel of Protein Design. Annual Review of
754 Biochemistry, Vol 79 79, 591-617.

755 Montecucco, C., 1986. How do tetanus and botulinum toxins bind to neuronal membranes.
756 Trends Biochem. Sci. 11, 315-317.

757 Montecucco, C., Rossetto, O., Schiavo, G., 2004. Presynaptic receptor arrays for clostridial
758 neurotoxins. Trends Microbiol. 12, 442-446.

Montecucco, C., Schiavo, G., Pantano, S., 2005. SNARE complexes and neuroexocytosis: how many, how close? Trends Biochem. Sci. 30, 367-372.

Moreno-Igoa, M., Calvo, A.C., Ciriza, J., Munoz, M.J., Zaragoza, P., Osta, R., 2012. Non-viral gene delivery of the GDNF, either alone or fused to the C-fragment of tetanus toxin protein, prolongs survival in a mouse ALS model. Restor. Neurol. Neurosci. 30, 69-80.

Moreno-Igoa, M*., et al.*, 2010. Fragment C of tetanus toxin, more than a carrier. Novel perspectives in non-viral ALS gene therapy. J. Mol. Med. (Berl.) 88, 297-308.

Muraro, L., Tosatto, S., Motterlini, L., Rossetto, O., Montecucco, C., 2009. The N-terminal half of the receptor domain of botulinum neurotoxin A binds to microdomains of the plasma membrane. Biochem. Biophys. Res. Commun. 380, 76-80.

Oates, E.C*., et al.*, 2013. Mutations in BICD2 cause dominant congenital spinal muscular atrophy and hereditary spastic paraplegia. Am. J. Hum. Genet. 92, 965-973.

Ohka, S., Sakai, M., Bohnert, S., Igarashi, H., Deinhardt, K., Schiavo, G., Nomoto, A., 2009. Receptor-dependent and -independent axonal retrograde transport of poliovirus in motor neurons. J. Virol. 83, 4995-5004.

Olivan, S., Calvo, A.C., Rando, A., Herrando-Grabulosa, M., Manzano, R., Zaragoza, P., Tizzano, E.F., Aquilera, J., Osta, R., 2016. Neuroprotective Effect of Non-viral Gene Therapy Treatment Based on Tetanus Toxin C-fragment in a Severe Mouse Model of Spinal Muscular Atrophy. Front. Mol. Neurosci. 9, 76.

Oliveira, H., Fernandez, R., Pires, L.R., Martins, M.C., Simoes, S., Barbosa, M.A., Pego, A.P., 2010. Targeted gene delivery into peripheral sensorial neurons mediated by self-assembled vectors composed of poly(ethylene imine) and tetanus toxin fragment c. J. Control. Release 143, 350-358.

Ovsepian, S.V., Bodeker, M., O'Leary, V.B., Lawrence, G.W., Oliver Dolly, J., 2015. Internalization and retrograde axonal trafficking of tetanus toxin in motor neurons and trans-synaptic propagation at central synapses exceed those of its C-terminal-binding fragments. Brain Struct Funct 220, 1825-1838.

Papagiannopoulou, D., Vardouli, L., Dimitriadis, F., Apostolidis, A., 2016. Retrograde transport of radiolabelled botulinum neurotoxin type A to the CNS after intradetrusor injection in rats. BJU Int. 117, 697-704.

Peck, M.W*., et al.*, 2017. Historical Perspectives and Guidelines for Botulinum Neurotoxin Subtype Nomenclature. Toxins (Basel) 9. DOI: 10.3390/toxins9010038

Pirazzini, M., Azarnia Tehran, D., Leka, O., Zanetti, G., Rossetto, O., Montecucco, C., 2016. On the translocation of botulinum and tetanus neurotoxins across the membrane of acidic intracellular compartments. Biochim. Biophys. Acta 1858, 467-474.

Pirazzini, M., Azarnia Tehran, D., Zanetti, G., Lista, F., Binz, T., Shone, C.C., Rossetto, O., Montecucco, C., 2015. The thioredoxin reductase--Thioredoxin redox system cleaves the

796 interchain disulphide bond of botulinum neurotoxins on the cytosolic surface of synaptic
797 vesicles. Toxicon 107, 32-36.

798 Pirazzini, M.*, et al.*, 2014. Thioredoxin and its reductase are present on synaptic vesicles,
799 and their inhibition prevents the paralysis induced by botulinum neurotoxins. Cell Rep 8,
800 1870-1878.

801 Pirazzini, M., Rossetto, O., Eleopra, R., Montecucco, C., 2017. Botulinum Neurotoxins:
802 Biology, Pharmacology, and Toxicology. Pharmacol. Rev. 69, 200-235.

803 Restani, L., Giribaldi, F., Manich, M., Bercsenyi, K., Menendez, G., Rossetto, O., Caleo, M.,
804 Schiavo, G., 2012a. Botulinum Neurotoxins A and E Undergo Retrograde Axonal Transport
805 in Primary Motor Neurons. PLoS Pathog. 8, e1003087.

806 Restani, L., Novelli, E., Bottari, D., Leone, P., Barone, I., Galli-Resta, L., Strettoi, E., Caleo,
807 M., 2012b. Botulinum Neurotoxin A Impairs Neurotransmission Following Retrograde
808 Transynaptic Transport. Traffic 13, 1083-1089.

809 Rinaldi, S., Brennan, K.M., Goodyear, C.S., O'Leary, C., Schiavo, G., Crocker, P.R., Willison,
810 H.J., 2009. Analysis of lectin binding to glycolipid complexes using combinatorial glycoarrays.
811 Glycobiology 19, 789-796.

812 Rossetto, O., Seveso, M., Caccin, P., Schiavo, G., Montecucco, C., 2001. Tetanus and
813 botulinum neurotoxins: turning bad guys into good by research. Toxicon 39, 27-41.

814 Rossor, A.M.*, et al.*, 2015. Phenotypic and molecular insights into spinal muscular atrophy
815 due to mutations in BICD2. Brain 138, 293-310.

816 Roux, S., Saint Cloment, C., Curie, T., Girard, E., Mena, F.J., Barbier, J., Osta, R., Molgo, J.,
817 Brulet, P., 2006. Brain-derived neurotrophic factor facilitates in vivo internalization of tetanus
818 neurotoxin C-terminal fragment fusion proteins in mature mouse motor nerve terminals. Eur.
819 J. Neurosci. 24, 1546-1554.

820 Rummel, A., 2013. Double receptor anchorage of botulinum neurotoxins accounts for their
821 exquisite neurospecificity. Curr. Top. Microbiol. Immunol. 364, 61-90.

822 Rummel, A., 2016. Two Feet on the Membrane: Uptake of Clostridial Neurotoxins. Curr. Top.
823 Microbiol. Immunol.

824 Rummel, A., Bade, S., Alves, J., Bigalke, H., Binz, T., 2003. Two carbohydrate binding sites
825 in the H_{CC}-domain of tetanus neurotoxin are required for toxicity. J. Mol. Biol. 326, 835-847.

826 Rummel, A.*, et al.*, 2007. Identification of the protein receptor binding site of botulinum
827 neurotoxins B and G proves the double-receptor concept. Proc. Natl. Acad. Sci. U. S. A. 104,
828 359-364.

829 Rummel, A.*, et al.*, 2009. Botulinum neurotoxins C, E and F bind gangliosides via a
830 conserved binding site prior to stimulation-dependent uptake with botulinum neurotoxin F
831 utilising the three isoforms of SV2 as second receptor. J. Neurochem. 110, 1942-1954.

832 Salinas, S., Bilsland, L.G., Henaff, D., Weston, A.E., Keriel, A., Schiavo, G., Kremer, E.J.,
833 2009. CAR-associated vesicular transport of an adenovirus in motor neuron axons. PLoS
834 Pathog. 5, e1000442.

835 Schafer, M.K., Bellouze, S., Jacquier, A., Schaller, S., Richard, L., Mathis, S., Vallat, J.M.,
836 Haase, G., 2017. Sensory neuropathy in progressive motor neuronopathy (pmn) mice is
837 associated with defects in microtubule polymerization and axonal transport. Brain Pathol. 27,
838 459-471.

839 Schiavo, G., Greensmith, L., Hafezparast, M., Fisher, E.M., 2013. Cytoplasmic dynein heavy
840 chain: the servant of many masters. Trends Neurosci. 36, 641-651.

841 Schiavo, G., Papini, E., Genna, G., Montecucco, C., 1990. An intact interchain disulfide bond
842 is required for the neurotoxicity of tetanus toxin. Infect. Immun. 58, 4136-4141.

843 Schiavo, G., Poulain, B., Rossetto, O., Benfenati, F., Tauc, L., Montecucco, C., 1992a.
844 Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease
845 activity depend on zinc. EMBO J. 11, 3577-3583.

846 Schiavo, G., Rossetto, O., Santucci, A., DasGupta, B.R., Montecucco, C., 1992b. Botulinum
847 neurotoxins are zinc proteins. J. Biol. Chem. 267, 23479-23483.

848 Schiavo, G., Shone, C.C., Bennett, M.K., Scheller, R.H., Montecucco, C., 1995. Botulinum
849 neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of
850 syntaxins. J. Biol. Chem. 270, 10566-10570.

851 Schiavo, G., Shone, C.C., Rossetto, O., Alexander, F.C., Montecucco, C., 1993. Botulinum
852 neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. J. Biol.
853 Chem. 268, 11516-11519.

854 Schmieg, N., Menendez, G., Schiavo, G., Terenzio, M., 2014. Signalling endosomes in
855 axonal transport: travel updates on the molecular highway. Semin. Cell Dev. Biol. 27, 32-43.

856 Schmitt, A., Dreyer, F., John, C., 1981. At least three sequential steps are involved in the
857 tetanus toxin-induced block of neuromuscular transmission. Naunyn Schmiedebergs Arch.
858 Pharmacol. 317, 326-330.

859 Simpson, L.L., 2000. Identification of the characteristics that underlie botulinum toxin
860 potency: implications for designing novel drugs. Biochimie 82, 943-953.

861 Sleigh, J.N.*, et al.*, 2017a. Trk receptor signaling and sensory neuron fate are perturbed in
862 human neuropathy caused by Gars mutations. Proc. Natl. Acad. Sci. U. S. A. 114, E3324-
863 E3333.

864 Sleigh, J.N., Vagnoni, A., Twelvetrees, A.E., Schiavo, G., 2017b. Methodological advances in
865 imaging intravital axonal transport. F1000Res 6, 200.

866 Stevens, R.C., Evenson, M.L., Tepp, W., DasGupta, B.R., 1991. Crystallization and
867 preliminary X-ray analysis of botulinum neurotoxin type A. J. Mol. Biol. 222, 877-880.

868 Stoeckel, K., Schwab, M., Thoenen, H., 1977. Role of gangliosides in the uptake and
869 retrograde axonal transport of cholera and tetanus toxin as compared to nerve growth factor
870 and wheat germ agglutinin. Brain Res. 132, 273-285.

871 Sugawara, Y., Yutani, M., Amatsu, S., Matsumura, T., Fujinaga, Y., 2014. Functional
872 Dissection of the Clostridium botulinum Type B Hemagglutinin Complex: Identification of the
873 Carbohydrate and E-Cadherin Binding Sites. PLoS One 9, e111170.

874 Swaminathan, S., Eswaramoorthy, S., 2000. Structural analysis of the catalytic and binding
875 sites of Clostridium botulinum neurotoxin B. Nat. Struct. Biol. 7, 693-699.

876 Takamizawa, K., Iwamori, M., Kozaki, S., Sakaguchi, G., Tanaka, R., Takayama, H., Nagai,
877 Y., 1986. TLC immunostaining characterization of Clostridium botulinum type A neurotoxin
878 binding to gangliosides and free fatty acids. FEBS Lett. 201, 229-232.

879 Terenzio, M., Golding, M., Russell, M.R., Wicher, K.B., Rosewell, I., Spencer-Dene, B., Ish-
880 Horowicz, D., Schiavo, G., 2014a. Bicaudal-D1 regulates the intracellular sorting and
881 signalling of neurotrophin receptors. EMBO J. 33, 1582-1598.

882 Terenzio, M., Golding, M., Schiavo, G., 2014b. siRNA screen of ES cell-derived motor
883 neurons identifies novel regulators of tetanus toxin and neurotrophin receptor trafficking.
884 Front. Cell. Neurosci. 8, 140.

885 Toivonen, J.M., Olivan, S., Osta, R., 2010. Tetanus toxin C-fragment: the courier and the
886 cure? Toxins (Basel) 2, 2622-2644.

887 Tsukamoto, K., Kohda, T., Mukamoto, M., Takeuchi, K., Ihara, H., Saito, M., Kozaki, S.,
888 2005. Binding of Clostridium botulinum type C and D neurotoxins to ganglioside and
889 phospholipid. Novel insights into the receptor for clostridial neurotoxins. J. Biol. Chem. 280,
890 35164-35171.

891 Udwadia, F.E., 1994. Tetanus. Oxford University Press, Bombay.

892 Vale, R.D., 2003. The molecular motor toolbox for intracellular transport. Cell 112, 467-480.

893 van Ermengem, E., 1979. Classics in infectious diseases. A new anaerobic bacillus and its
894 relation to botulism. E. van Ermengem. Originally published as "Ueber einen neuen
895 anaeroben Bacillus und seine Beziehungen zum Botulismus" in Zeitschrift fur Hygiene und
896 Infektionskrankheiten 26: 1-56, 1897. Rev. Infect. Dis. 1, 701-719.

897 Wade, A., Thomas, C., Kalmar, B., Terenzio, M., Garin, J., Greensmith, L., Schiavo, G.,
898 2012. Activated leukocyte cell adhesion molecule modulates neurotrophin signaling. J.
899 Neurochem. 121, 575-586.

900 Wang, T.*, et al.*, 2016. Flux of signalling endosomes undergoing axonal retrograde transport
901 is encoded by presynaptic activity and TrkB. Nat Commun 7, 12976.

902 Wang, T.*, et al.*, 2015. Control of autophagosome axonal retrograde flux by presynaptic
903 activity unveiled using botulinum neurotoxin type a. J. Neurosci. 35, 6179-6194.

Weisemann, J., Stern, D., Mahrhold, S., Dorner, B.G., Rummel, A., 2016. Botulinum neurotoxin serotype a recognizes its protein receptor SV2 by a different mechanism than botulinum neurotoxin B synaptotagmin. Toxins (Basel) 8.

Wiegand, H., Erdmann, G., Wellhoner, H.H., 1976. 125I-labelled botulinum A neurotoxin: pharmacokinetics in cats after intramuscular injection. Naunyn Schmiedebergs Arch. Pharmacol. 292, 161-165.

Williamson, L.C., Bateman, K.E., Clifford, J.C., Neale, E.A., 1999. Neuronal sensitivity to tetanus toxin requires gangliosides. J. Biol. Chem. 274, 25173-25180.

Yao, G., Lee, K., Gu, S., Lam, K.H., Jin, R., 2014. Botulinum neurotoxin A complex recognizes host carbohydrates through its hemagglutinin component. Toxins (Basel) 6, 624-635.

Yeh, F.L., Dong, M., Yao, J., Tepp, W.H., Lin, G.Y., Johnson, E.A., Chapman, E.R., 2010. SV2 Mediates Entry of Tetanus Neurotoxin into Central Neurons. PLoS Pathog. 6, e1001207.

Zanetti, G., Azarnia Tehran, D., Pirazzini, M., Binz, T., Shone, C.C., Fillo, S., Lista, F., Rossetto, O., Montecucco, C., 2015. Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism. Biochem. Pharmacol. 98, 522-530.

Zhang, S.*, et al.*, 2017. Identification and characterization of a novel botulinum neurotoxin. Nat Commun 8, 14130.

Zhang, Y., Varnum, S.M., 2012. The receptor binding domain of botulinum neurotoxin serotype C binds phosphoinositides. Biochimie 94, 920-923.

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.

Figure 1

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Figure 1